Predicting toxic effects of contaminants in ecosystems using single species investigations

Habilitationsschrift

vorgelegt dem
Fachbereich Biologie/Chemie
der Universität Bremen

von
Rolf Altenburger

Leipzig
März 2002

"Wovon man nicht sprechen kann, darüber muß man schweigen."

L. Wittgenstein
Tractatus logico-philosophicus

Gliederung/Structure

der Habilitationsschrift:

"Predicting toxic effects of contaminants in ecosystems using single species investigations"

I Summary

II Bioassay-directed identification of organic toxicants in river sediment in the industrial region of Bitterfeld (Germany) - A contribution to hazard assessment.
Werner Brack, Rolf Altenburger, Uwe Ensenbach, Monika Möder, Helmut Segner und Gerrit Schüürmann
Archives of Environmental Contamination and Toxicology, 37, 164-174. (1999)

III Brauchen wir einen Biotest mit höheren Pflanzen in der aquatischen Toxikologie?
Beata Praszczyk, Rolf Altenburger, Jörg Oehlmann, Bernd Markert und Gerrit Schüürmann
In: J. Oehlman, B. Markert (Hrsg.). Ökotoxikologie. Ökosystemare Ansätze und Methoden. Landsberg: ecomed. S. 151-163. (1999)

IV Exposition und Toxizität – Der Faktor Zeit in Routinebiotests
Rolf Altenburger und Thomas Backhaus
In: W. Mücke, M. Link (Hrsg). *Biotests in der Praxis*, Institut für Toxikologie und Umwelthygiene, Technische Universität München. S. 61-74. (2000)

V Predictability of the toxicity of multiple chemical mixtures to *Vibrio fischeri*: Mixtures composed of similarly acting chemicals
Rolf Altenburger, Thomas Backhaus, Wolfgang Boedeker, Michael Faust, Martin Scholze und L. Horst Grimme
Environmental Chemistry and Toxicology, 19, 2341-2347. (2000)

VI Quantitative structure–activity analysis of the algae toxicity of nitroaromatic compounds
Heike Schmitt, Rolf Altenburger, Bernd Jastorff und Gerrit Schüürmann
Chemical Research in Toxicology, 13, 441-450. (2000)

VII Alterations of physiological energetics, growth and reproduction of *Daphnia magna* under toxicant stress.
Monika Knops, Rolf Altenburger und Helmut Segner
Aquatic Toxicology, 53, 79-70. (2001)

VIII Mixture Toxicity of Priority Pollutants at No Observed Effect Concentrations (NOECs)
Helge Walter, Federica Consolaro, Paola Gramatica, Martin Scholze und Rolf Altenburger
Ecotoxicology, accepted

IX Erfolgskontrolle bei Grundwasser-Sanierungen: Wird die Toxizität durch Behandlung reduziert ?
Rolf Altenburger, Eberhard Küster, Falk Dorusch, Bettina Meißner, Holger Weiß und Gerrit Schüürmann
Grundwasser, eingereicht

X Algal Toxicity of Nitrobenzenes – Combined Effect Analysis as a Pharmacological Probe for Similar Mode of Action
Rolf Altenburger, Heike Schmitt und Gerrit Schüürmann, in Vorbereitung

Seitenangaben zu den Kapitel **Seite**

I	**Summary "Predicting toxic effects of contaminants in ecosystems using single species investigations"**	**7**
II	**Bioassay-directed identification of toxicants**	**61**
III	**Brauchen wir einen Biotest mit höheren Pflanzen?**	**75**
IV	**Der Faktor Zeit in Routinebiotests**	**95**
V	**Predictability of the toxicity of chemical mixtures**	**111**
VI	**Quantitative structure–activity analysis of nitroaromatic algal toxicity**	**121**
VII	**Energetics of *Daphnia magna* under toxicant stress**	**141**
VIII	**Mixture Toxicity at NOEC**	**155**
IX	**Toxizitätsreduktion als Sanierungserfolgskontrolle**	**173**
X	**Combined Effect Analysis as a Pharmacological Probe**	**191**

Kapitel I - Summary zur Habilitationsschrift

Predicting toxic effects of contaminants in ecosystems using single species investigations

Contents

Abstract

1 Introducing the context

2 Frame for prediction activities

- Which chemicals to address?
- What are adequate time and spatial scales?
- What are appropriate criteria for assessment?

3 Methodological considerations

3.1 Ecosystem description
3.2 Effect and mode of action analysis

4 Methods and tools

4.1 Concentration-response relationships
4.2 Time-response relationships
4.3 Bioassay-directed fractionation and identification of toxicants
4.4 Effect analysis
4.5 Combined effect analysis
4.6 Quantitative structure-activity relationship

5 Scope for predictions

5.1 In situ-toxicity testing
5.2 Single species sensitivity distributions
5.3 Comparison of responses from single and multispecies testing
5.4 Ecological considerations
5.5 What is the question?

Abstract

The usefulness of information gained from investigations of single species for predicting adverse effects of chemical contamination on aquatic ecosystems is discussed in this contribution. The frame for prediction efforts is thereby provided by the type and number of chemicals considered, the time and spatial scale of pollution and the criteria for an assessment. Further, any attempt to predict ecotoxicological effects on the basis of single species observation requires to instrumentalize current understanding of ecosystems and biological action of compounds for a specific assessment task.

Various methods and tools that link chemical and biological type of information to specifically establish a functional relationship between exposure concentration and biological effect, model biological responses of long-term exposure, identify components of ecotoxicological concern in complex contaminated samples, calculate expected combined effects for mixtures of pollutants, understand modes of ecotoxic action, and predict biological activities from compounds structural properties are reviewed. The methodologies introduced all have their specific drawbacks concerning the scope to assess and predict ecosystem responses. Various approaches addressing extrapolation problems like *in situ*-toxicity testing, species sensitivity distributions, comparative studies using laboratory assays and micro- and mesocosm studies are additionally considered.

It is concluded that all evidence so far shows, that single species data on the toxicity of pollutants can be used to predict the potential of adverse effects in ecosystems. There is no evidence that complex model ecosystems are systematically more or less sensitive to toxicants than single species tests. Principal limitations for extrapolation emerge when longer time scales are of concern or when structures or processes above the level of populations are affected. Additionally, ecological issues considered from a recovery perspective like recolonisation or functional replacement of species might modify assessment views.

1 Introducing the context

The protection of the environment has become an ultimate political goal and social value in industrialised countries in the early 1970ies as documented with the United Nations Conference on the Human Environment held in Stockholm, Sweden in 1972 (Halpern, 1993). This process developed in scope from a mere conservationist point of view to a sustainability-oriented approach with the Rio Declaration in 1992 (Anon, 1992). Ever since the beginning raise in popular awareness that human activities and in particular the management of material flows may impair the human environment, sciences have been asked to assess and predict the consequences of releasing all sorts of chemicals into the environment. This perception can be traced e.g. when studying legal requirements regarding the protection of the environment that have considerably increased throughout the last three decades. They almost uniformly at one stage require scientific or expert judgement of anticipated deleterious effects. Various lines of discussion in policy defined legislative activities may be distinguished. For a historical perspective on this the reader may refer to e.g. Milles (1989, 1991). From a decision process perspective one may label the different purposes as hazard identification, hazard assessment, risk characterisation, risk assessment and risk management (OECD, 1995). The reader has to be aware, however, that there is no uniform understanding of any of the terms employed. Key wording regarding the scope of intended environmental protection as defined in specific laws are e.g. "no harmful effect on human or animal health, directly or indirectly (e.g. through drinking water, food or feed) or on groundwater; [...] no unacceptable influence on the environment" (EEC, 1991).

Transforming political and juridical concepts into regulatory and administrative work is a challenge in itself. Various scopes like predictive assessments required for industrial chemicals or active ingredients of drugs or pesticides are to be separated from retrospective judgements on the effects of effluent discharges into rivers or emissions to the air. Site- and time-specific evaluations like evaluating run-off from waste disposal sites may be separated from utilisation specific approaches. As examples for the latter the formulation of water quality objectives for the protection of fishing stocks or the preservation of aquatic biocoenosis may be named. To support the setting of procedures and standards that are likely to hold even in cases of legal controversy and challenge, whole groups of experts at various national and international fora (like DIN, AFNOR; SIS, BSO, ASTM, OECD; CEN; ISO; SETAC) discuss the definition of protocols for any assessment to be made.

When sciences were being ascribed to deliver rational approaches to assess and predict adverse effects of chemicals on the environment, a whole new branch called ecotoxicology emerged from the collaboration of several biological and chemical subdisciplines. Like in the medical sciences when considering the developments in pharmacology and toxicology, several lines of reasoning developed in ecotoxicology. They range from questions of identifying mechanisms of action, to understanding of translation of effects from molecular interactions to responses in the structure or function of an ecosystem. Also, directions of research vary greatly covering more academic questions of generic principles of interactions

or rather applied aspects like the identification of remediation priorities or the management of a waste dump site.

This brief outline of the various activities of different stakeholders in the field hopefully enlightened the complex setting of expectations when writing about assessment and prediction for ecosystems. In no way a pure academic thinking on understanding ecosystems structure and function may prevail nor is a mere orientation on pragmatic issues like defining rules for discharge fees appropriate. The assessment of chemical effects in the environment is a demand from the general public linked with frameworks and values concerning what to consider and what to protect deriving from various political and economic backgrounds. However, the development of rational methodology and procedures for performance of those societal choices is the task of scientists. Not surprisingly these goals are often too ambitious to be met by simple and universal solutions. Instead pragmatic tools, rational choices and refined methodologies are being developed in response to specific tasks. In order to make good use of available knowledge and instruments, it is therefore vital to understand the scope and limitations of our poor trials.

Assessment and prediction of effects of contamination on ecosystems commonly relies on consideration of chemical exposure i.e. identifying targets at risk, determining an exposure concentration with respect to the bioavailability of pollutants in a specific environmental milieu and assessing biological responses. The scope of this article within the context of this book is focussed to highlight and reflect the differences in scientific approaches using single species as qualitative and quantitative indicators for predicting adverse effects of chemical contamination on aquatic ecosystems.

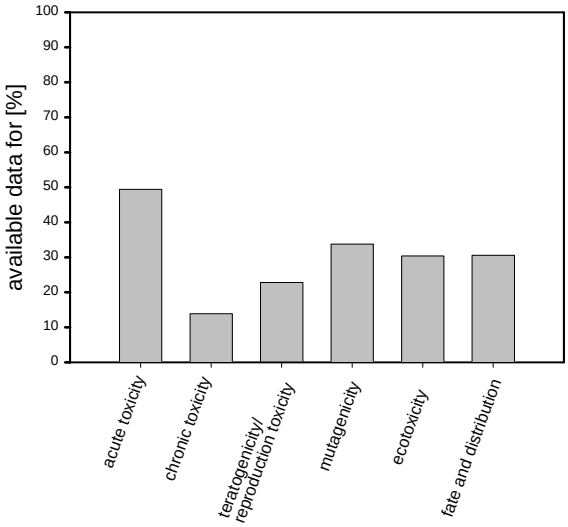
2 Frame for prediction activities

Apart from the chemical and biological issues of how to perform an assessment and predict effects of pollutants for ecosystems which will be considered in the subsequent parts of this contribution, there are factors that define a frame for any kind of prediction exercises, which have to be reflected. In particular, we have to deal with (i) a multitude of chemicals being present in the environment, (ii) time and scale of a pollution situation, (iii) definition of criteria for an assessment.

Which chemicals to address?

The chemical abstract service (CAS) in 2001 counted over 18 million organic and inorganic substances, of which more than 2 millions were commercially available chemicals (http://info.cas.org/cgi-bin/regreport.pl). There is no way to handle these compounds on a one by one basis in any kind of hazard assessment. In view of this, many national and international authorities have begun to build different types of chemical inventories (European Inventory of Existing Commercial Chemical Substances - EINECS, or the Toxic

Release Inventory - TRI) to select compounds for prior assessment. One approach in several OECD countries was to identify and list so-called high production volume chemicals (HPVCs), namely chemicals that are produced or traded in amounts exceeding 1000 t/a. The EEC regulation (EEC, 1990) counted about 2000 of such substances regarded as priority compounds for environmental risk assessment.



The US-American Environmental Protection Agency (EPA) published a survey (Betts, 1998) on the availability of basic toxicological data for such long existing and traded chemicals of high production volumes (fig. 1). It shows that for the majority even of priority chemicals there is a great lack of basic information typically needed for any normative hazard assessment.

Figure 1: Availability of data from experimental studies for environmental risk assessment (modified after Betts, 1998)

Besides, whole chemical groups of high public or toxicological concern like dioxins or pesticides are not covered. Moreover, the underlying problem is that for any given amount of resources for hazard and risk assessment activities, decisions have to be taken on the allocation of efforts which often leads to the question, do we want to focus on many compounds with little depth of knowledge or on just a few chemicals with great depth of understanding?

What are adequate time and spatial scales?

Occurrence of contaminants in the environment may vary greatly with respect to range, sequence and duration. Also in terms of management options exposure may be considered as accidental or unavoidable, as point source-related or diffuse. Similarly, type, extension in space and development of biosystems at risk show high variability. The scope for predictions is thus very much dependent on fixing borderlines in time and space. These in turn very often derive from specific demands for an assessment. A prospective evaluation of the potential of a new pesticide for e.g., investigated for admission, to affect invertebrate life by spraydrift to riverine systems, is to be distinguished from a retrospective assessment such as contaminants of a sediment investigated for suitability to be used as land fill material.

In assessing chemicals in the environment, discrete events (short-term) have to be distinguished form long-term exposure. Exposure over longer periods may cause a change of the abiotic conditions and may lead to adaptation processes in the ecosystem. Both exposure scenarios (long-term; short-term) interfere at different levels of an ecosystem and must be evaluated by on different scales of time and space.

Because of the natural heterogeneity of an ecosystem, small scale effects are more difficult to detect than large scale events. On the other hand, short and ”small” events are probably more frequent than disasters with large scale effects. E.g. the Sandoz accident at the river Rhine provoked a total break down of the ecosystem (fish kill) but small but frequent effluents of a toxicant into a stream may remain unidentified because of the small scale of effects, e.g. changes in the microbenthic community. These small scale effects need more precise investigation methods to detect changes in the ecosystem.

Further, the scale of abiotic ecosystem features must be taken into consideration, when assessing effects of a contamination in this ecosystem. E.g., aquatic ecosystems are characterised by a great variation of residence time of water, influencing persistence, degradation or accumulation of chemicals in the ecosystem. In running waters, toxic effluents are transported downstream, so a spatial discrepancy of the point of release and observed effects may be found. In standing waters, the toxicants may rest in the water body for a long time, translocated and bound to the sediments. These may be periodically resuspended e.g. by annual circulations of a mictic lake, so effects may be detected for a longer time period.

The mobility or space of colonisation of a population is further of importance, when assessing the scale of a contamination. For example in streams and rivers, macrozoobenthic organisms, exposed to a short pulse of a toxicant can escape into the hyporheic interstitial, an ecotone, which exists in the loose sediments between the stream bed and the aquifer. From this refugium, they can recolonize the stream quite fast. Smaller and immobile organisms, e.g. the meiobenthos, organisms of the biofilm, or macrophytes are not able to avoid the exposure. Disturbances may be detectable much better within these groups of organisms than by investigating species with the potential of migration and recolonisation.

What are appropriate criteria for assessment?

Imagine a regionally contaminated aquifer which has to be remediated. Ecotoxicologists are requested to provide tools that allow assessments of the treated groundwater and comparisons of different technological options (e.g. the SAFIRA project, http://safira.pro.ufz.de/) with respect to the protection of ecosystems. Commonly, faced with this question one would call for terms of reference by asking back: What type of biosystems are to be protected?; What is to be regarded as a significant adverse effect?; And what in turn seems to be an acceptable hazard? An elaboration of these issues may be found in Forbes and Forbes (1994) and essentially they are a reflection that criteria for ecotoxicological assessment are *eo ipso* not scientific. Once the terms of reference have

been defined scientific rationales may proceed. In the above example we may wish to conserve the fish population in the nearby freshwater for recreational purposes or we want to protect the microbial functions of the linked waste water treatment plant as a biological service. Another goal could be to avoid adverse effects on the interstitial faunistic community with stygophile and stygobite life forms in the adjacent groundwater as a biodiversity protection measure. Given the necessary resources are available, each of the protection goals can be addressed using specific models, biotests and expertise. However, none of the specific prediction instruments developed would allow extrapolative use for any of the others. Furthermore, additional requirements may have to be met in an assessment exercise, like that the assessment has to be made very fast to allow adequate intervention, or it may be asked for data that hold in a legal challenge.

3 Methodological considerations

Once the context of an ecotoxicological assessment or prediction exercise is defined, biological thinking prevails. Developing or utilising biological tools for specific purposes should regard what is known with respect to the description of structure and function of ecosystems and how we do describe modes of toxic interference. Both aspects strive to instrumentalize current understanding of ecosystems and biological action for a specific assessment task. For the considerations of this overview, the methodological questions behind the aim to link single species observation to ecosystem effects basically are: What can be put under observation? What can be modelled in most simplistic ways?

3.1 Ecosystem description

The major challenge in ecotoxicology thereby is to link measured endpoints of a single species under conditions as strictly defined as possible to ecological assessment endpoints, including communities and ecosystem structure and function.

There are various parameters that may be put under observation to characterise survival, growth, behaviour or development of single species or population dynamics (growth rate, death rate, density etc.). Effects of toxicants can be quantified in the laboratory for these parameters, and used to assess direct effects of toxicants on the organism. Parameters of the population dynamic of a species may even be inserted in models, which try to simulate (predict) effects on a multispecies or ecosystem level.

On the other hand, all parameters, belonging specifically to a community level (species composition, species distribution, intra- and interspecific interactions) cannot be derived from a single species level. Effects of toxicants on these endpoints, which are essential in regulating community answers to toxicants cannot be observed in such test approaches.

Further, ecosystems are strongly influenced by abiotic factors (temperature, pH, matrix effects etc). These parameters also influence speciation, bioavailability and so the effects of a substance on a community in an exposed ecosystem. These interactions between pollutants, environmental milieu factors and biosystems may be investigated in a laboratory single species test in a restricted way. In a simple reaction chain, represented by one or a very few abiotic factors, substances and species, some causal connections can be derived and eventually modelled. The whole complexity of an ecosystem, however, can hardly be described. Table 1 lists characteristics unique to different levels of biological complexity and parameters that may be accessible for toxicological consideration.

Another aspect is the composition and diversity of species to be found in a community and its relation to chemical effects. There is no hierarchy in sensitivity of species, which could be easily generalised. However, it is not possible to test all species of an (exposed) ecosystem for each chemical. Established single species test systems are mainly composed of ubiquists, which have turned out to be suitable for a good (reproducible) test situation and are easy to cultivate. In the ecosystem, specialists (stenoec species) are adapted to their environment, which characterise the ecosystem. These species are often rare (lists of endangered species) and sometimes characterised by complicated life cycles, tightly connected to the ecosystem characteristics. These species could be more sensitive towards chemical exposure and in many cases their elimination will be more difficult to overcome than for robust species.

Table 1: Biological levels of organisation and toxicological observation of specific interferences

level	**test system**	**endpoints**	**information**
organism	single species test	survival, growth, behaviour, physiological parameters, scope for growth	direct impact on the organism, species sensitivity, mode of action
population	single species test	growth / death rate, density, distribution	parameters of population dynamics, intraspecific interaction
2-4 species	multispecies test	scope for growth, grazing rates (loss rates), competition	interspecific interaction
community	community test	species composition / distribution, diversity, succession	interspecific interaction, tolerance, adaptation, invasion, exclusion of species
ecosystem	microcosm, mesocosm, enclosures, field studies	energy flow, food web, distribution of the toxicant	impact of abiotic ecosystem parameters ecosystem structure, function bioavailability

The niche of stenoec species will subsequently be occupied by an ubiquist resulting in a loss of diversity. These mechanisms might be more drastic in a sensitive ecosystem (e.g. bogs, springs) with many stenoeic species than in anthropogenic landscapes (e.g. agricultural

landscape), which are disturbed by other factors already (structural changes etc.) and characterised by a small variety of ubiquists.

In summary, the above arguments show that in order to make useful contributions to an ecological assessment and prediction of chemical effects on the basis of single species testing, information regarding alterations with respect to behaviour, individual growth, mortality and reproductive success is needed using species with a well-understood ecology.

3.2 Effect and mode-of-action analysis

The second methodological consideration concerns the description of biological effects and the understanding of modes of toxic action. Knowledge of the mode of action of a chemical means to understand the interrelations of all observable effects provoked by the defined amount of a chemical in a biosystem. This of course is not much easier than describing an ecosystem in the first place. The most crucial point for our purpose therefore is the reflection of the effect to be put under observation. It might be helpful to distinguish between three points: firstly, the effect parameters as the biological structure or function which gains the focus like reproduction; secondly, the observation technique which is employed, like the photometric measurement of a suspension's light scattering, under a defined protocol, and thirdly the derived effect descriptors like an EC_x which is the estimated concentration (or dilution respectively) of a chemical that is predicted to elicit a certain response.

Using single organisms instead of whole ecosystems as instruments for assessing the status of the environment or pollution effects has a tradition in itself. The first indicator systems were established to assess the nutrient status of organically polluted water bodies. In order to obtain an indication of the nutrient status of a site of interest the observation of the occurrence and abundance of species at sampling sites were linked to knowledge on the ecophysiological characteristics of indicator species. From there bioindication of pollutant effects that altered the occurrence or abundance of indicator species could be derived when relating site-specific observations to 'unpolluted' reference sites. Classical work has been performed by Kolkwitz (1902) and Kolkwitz and Marson (1950) who invented the system of an index of "Saprobie" in running waters. Assessment of air pollution effects in industrial landscapes using lichens (Kreeb, 1990) and of readily decomposable organic water pollution using macroinvertebrates in streams (Diamond and Daley, 2000) are well known current examples of these approaches. When the observation of single species was extended to regard the performance of individuals, biomonitoring and biotesting of adverse effects of chemicals became established. Both fields rapidly developed various techniques and applications as it was possible to perform most work in laboratories and thus applying methods that have been developed in physiological or biochemical research. An overview of the various biomonitoring strategies is provided by deZwart (1995), who categorises different fields such as toxicity monitoring of effluents, ambient toxicity monitoring, continuous biological monitoring, and ecosystem biomonitoring. In biotesting, environmental pollution is reduced to an environmental sample to be tested. Nusch (1992) gives an early account of

the various demands that can be raised and specifically addressed in biotesting. After two decades of bioindicator, biotest and biomonitor development and use, a few rationales can be distinguished that may claim a consented status regarding the principles of effect assessment based on experimental biological data:

- *pars pro toto*-principle, i.e. test protocols are used employing definite species which than act as representative of whole taxa or trophic levels;
- use of biotest batteries instead of a single test organism realising that there is no such thing as a most sensitive species;
- bioassay with optimised signal to noise ratio are used, by allowing only the chemical to provide a constraint on the effect parameter under observation while providing optimum for all other factors.

When prediction is the goal in effect assessment e.g. as it is the case in chemical hazard assessment, categorisation and modelling efforts become important in addition to the above described effect description tools. The methodologies developed for these purposes be it e.g. quantitative structure-activity relationships or physiologically based pharmacokinetic modelling do need some principal understanding regarding the interaction of pollutants with biosystems (Escher and Hermens, submitted). Principal understanding of toxic effects may derive from identification of primary molecular targets, biochemical studies of primary actions, or physiological and histological description of the following alterations. While the primary interaction may be referred to as mechanisms of action, toxic action is a process requiring the translation of functional or structural effects to response levels relevant for organismic performance, which is often referred to as mode of action.

In essence, when trying to predict ecosystem effects on basis of single species information the challenge of biodiversity translates into the effort to sufficiently represent different effect qualities that might be evoked from contamination of ecosystems with chemicals at high enough sensitivity. Finally, technical issues such as how to generate most precise and accurate information by regarding at the various sources of errors are discussed in the literature.

4 Methods and tools

Whenever the adverse effects of chemical contamination of ecosystems is to be predicted or to be assessed the challenge is to combine chemical and biological information i.e. to link analytical data on occurrence, identity and quantity of xenobiotics to information on adverse effects on biota. Very often these two types of information are produced in separated monitoring and surveillance efforts and are then handled as completely independent type of information. This leaves the prediction or assessment job in trouble of either speculating about the hazard potential of a compound that has been analysed on a particular date, for whatever reason or with little clue about the causes of an observed impairment of a biological function. In the following we will therefore place special emphasis on methods and tools that link chemical and biological type of information to specifically:

- establish a functional relationship between exposure concentration and effect;
- model biological responses of long-term exposure;
- identify components of ecotoxicological concern in a complex contaminated sample;
- calculate expected combined effects for mixtures of pollutants;
- understand modes of ecotoxic action; and
- predict biological activities from compounds structural properties.

4.1 Concentration-response relationships

For prediction purposes, exposure concentrations of considered pollutants or dicharges that are to be regarded as non-toxic for ecosystems are required in order to provide references for management activities. Risk management procedures for chemicals commonly rely on effect assessments based on single substance evaluations and on fixing of threshold values i.e. no effect concentrations (NECs) as a boarderline between an acceptable and an unacceptable risk. When asking scientific communities, this perception will immediately be translated into no observed effect concentrations (NOECs) or no observed adverse effect levels (NOAELs). To derive such values, statistical tests are used to compare the variances of a control and a treated situation and identify the highest test concentration that proves to be of no statistical difference from the control. For the last fourty years this concept has been a basis for regulating various chemicals, and it is still enshrined in various guidelines, standards and norms. However, as it is difficult for the experimentator to observe "no effects" and with acknowledging furthermore that there are severe drawbacks from a statistical point of view (details of the discussion e.g. in Laskowski, 1995, Chapman et al., 1996, Moore and Caux, 1997) there seems now consensus reached to move away from this predictive approach (for review see OECD, 1998). The shortcommings of a NOEC based assessment approach become evident when looking at the issue of mixtures as illustrated in Figure 2.

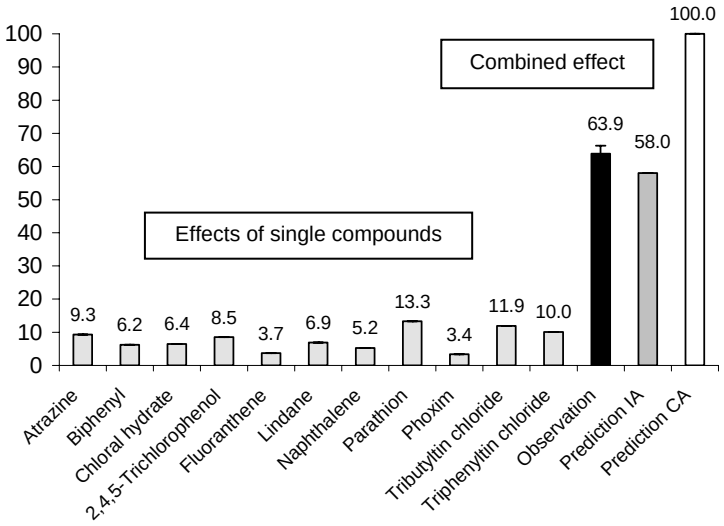


Figure 2: Observed and predicted mixture toxicity provoked from a multi-component mixture with the individual compounds present at NOEC concentration on algal reproduction (from Walter et al., accepted, see chapter V and VIII).

Instead an approach is favoured that focuses on a standing paradigm in toxicological research, namely that contaminant exposure an biological responses are functionally related.

The objectives of determining such concentration-response relationships using quantitative models are to allow

- reproducible derivation of characteristic values used in chemical risk assessment procedures like an EC50 (effect concentration at which 50 % of a specified effect is estimated to be evoked) or a LID (Lowest inhibitory dilution which produces a specified effect regarded as significant given a fixed dilution series of an environmental sample);
- comparison of compound properties in terms of intrinsic activity and effectiveness i.e. position and slope of a concentration-response curve;
- statistically valid predictions to low effect concentrations which are typical for many environmental contamination patterns.

Establishing a functional relationship between exposure concentrations of pollutants and biological effects requires experimentation using varying dilution often as geometric series in an appropriate range to observe varying responses of the effect put under observation. Figure 3 provides an example for the effect of various concentrations of the polyaromatic hydrocarbon naphthalene and its inhibitory effect on the reproduction of unicellular algae growing as a synchronous culture and being exposed for one generation cycle of 24 hrs (Walter at al., accepted).

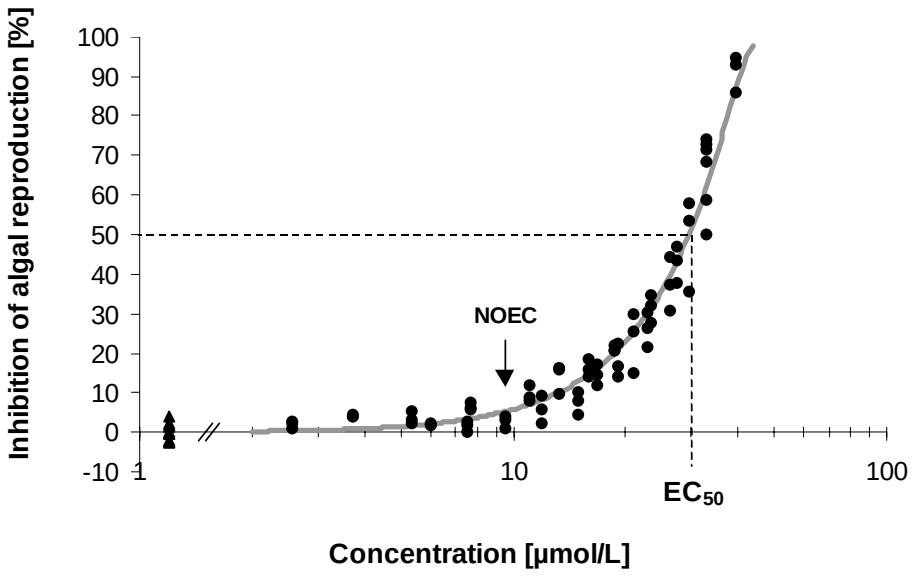


Figure 3: Concentration-response model and data for the naphthalene induced inhibition of reproduction of the green alga *Scenedesmus vacuolatus* (from Walter et al., accepted, see chapter VIII).

The experimentally determined data are then fed into appropriate biometrical models that by iterative procedures do calculate estimates for the model parameters. Plotting the estimated function against observed data (Figure 3) or residue analysis allows to assess the fit of the chosen function to the observed data using a probit model of the form:

$$\text{Effect} = \frac{1}{2\pi}\int_{-\infty}^{k(\text{conc.})} \exp(-u^2/2)du, k(\text{Conc.}) = \theta_1 + \theta_2 \frac{\text{Conc.}^{\theta_3} - 1}{\theta_3} = \text{Probit}\left(\theta_1 + \theta_2 \frac{\text{Conc.}^{\theta_3} - 1}{\theta_3}\right) \quad \textbf{(Eq. 1)}$$

The parameter estimates for this example were θ_1 = -85.44, θ_2 = 52.67, and θ_3 = 0.087. There are various models to choose from, most of which deliver differences only when regarding effect estimations for high or low effects (Christensen 1984, Moore and Caux, 1997, Shukla et al., 2000, Scholze et al., 2001).

Once a concentration-response relationship has been established it may be used to derive parameters like ECx values for various purposes. Active ingredients, purposefully released to the environment may be assessed comparatively concerning their unwanted effects. Holten Lützhoft et al. (1999) provide an example, comparing the phytotoxicity of seven antibacterial drugs applied in Danish fish farming using a cyanobacterium and two algal species as test organisms. Using estimated effect concentrations they were able to rank the different compounds according to their relative phytotoxicities. Further, they showed that the cyanobacterium *Microcystis aeruginosa* responded several orders of magnitude more sensitive compared with the eucaryotic plant species. This is easily understood considering the mode of action of the concerned compounds, which tend to be specific for interaction with procaryotic growth and reproduction processes.

An established concentration-response relationship may also be utilised to assess whether chemically detected amounts of a given pollutant sufficiently explain observable effects on organisms. Figure 3 provides an example for contaminated groundwater from the Bitterfeld area in Germany. Mass balances based on GC/MS-Screening showed that monochlorobenzene is the dominant contaminant for most ground water probes analysed in this particular area at the quarternary water table. Based on experimentally determined concentration-response functions for monochlorobenzene Figure 4 depicts the expected effects in *Vibrio fischeri* for chemically detected concentrations of chlorobenzene in different wells of the contaminated area. This expected effect is then compared with the observed toxicity of groundwater probes for the same organism. Obviously, chlorobenzene though present in high amounts, is not responsible for the observable toxicity. This can also be shown for the alga *Scenedesmus vacuolatus* and the crustacean *Daphnia magna*. The modelling of the toxicity of different probes for the contaminated area, however, provides a consistent description. Thus other components yet unidentified might add in mixture to the observable toxic effects.

(A) expected toxicity

(B) observed toxicity

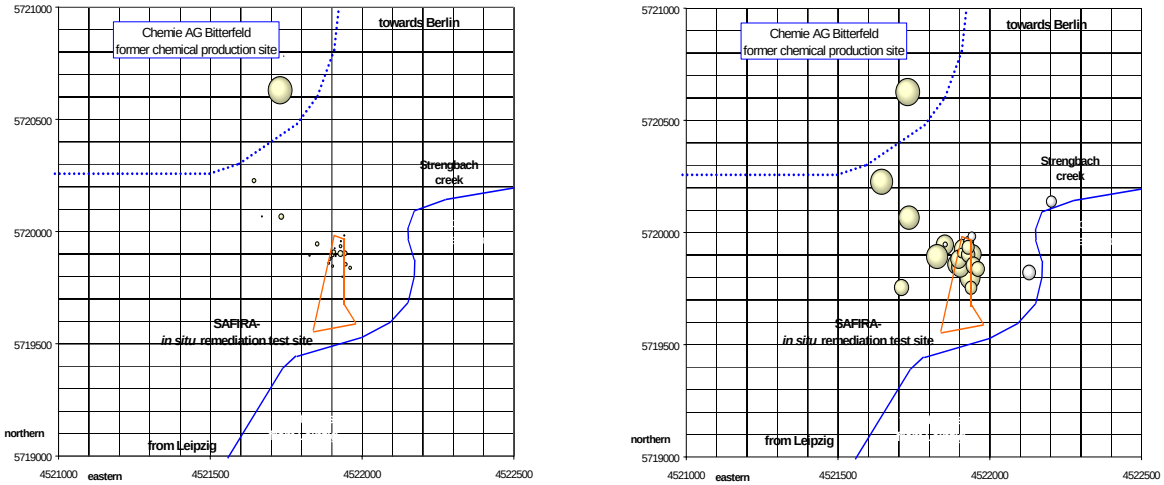


Figure 4: Map of expected (A) short-term bacterial toxicity in groundwater probes of a contaminated area, using analytically determined amounts and concentration-response relationships for chlorobenzene. (B) depicts the same area with observed bacterial responses towards groundwater probes. The bubble sizes on the maps indicate the degree of toxic response (see chapter IX).

Concerning the predictive scope of a concentration-response analysis there are several issues to be taken in mind. The duration of exposure as well as the time for effect propagation do have to be regarded with respect to the chosen effect parameter. A chronic effect e.g. has to be seen in relation to the life span of a particular organism. Most unfortunate is the discussion of sensitive endpoints that can very often be found in bioassay discussions. Bearing the objective of most investigations in mind, that there has to be some assessment or prediction for an ecosystem the requirements should be pretty evident. If there is no biological argument for a definite time of observation, like the completion of a generation cycle for instance, than there has to be a consideration of the time-response relationship (see 4.2). Furthermore, effects to be observed in experiments that are meant to relate to ecosystem assessments should strive to link observations to life table parameters or growth to allow ecological considerations of effects at the population level. Finally, an environmental concentration of a pollutant may not easily translate into an effective dose for an organism. The understanding of the often dynamic relationships between contaminant exposure and bioavailable concentrations are again research topics of their own right.

There are various techniques available to cope with dose estimations for unknown or fluctuating exposure situations which utilise cumulative responses, exposure history data, flow through exposure systems, or bioaccumulation biomonitors. The concepts of lethal body burdens (LBBs) and physiologically based toxicokinetic modelling (PBPK) (e.g. Yang et al. 2000) provide scope for refined dose estimations in organism-based hazard assessment. The functional description of concentration-response relationships though often employed offers many untapped potentials for the prediction of effects beyond cut-off values. This

includes contributions to the identification of modes of action (e.g. Altenburger et al., 1995) or the relevance of environmental milieu factors like pH for observable effects (Fahl et al., 1995).

4.2 Time-response relationships

The prediction of pollutant effects as a goal evidently is a time-related enterprise. Time in biological systems is an important variable e.g. regarding endogenous rhythms or different developmental stages of an individual; the age composition of a population or the succession state of an ecosystem to name a few. All these biological events in time have been shown to influence responses to chemical stress. Investigations addressing this most trivial fact explicitly, however, are not mainstream and a so-called endpoint discussion prevails instead. Even parameters intended to include time aspects like growth rates are often one point estimates. So whenever biological responses are not an end in themselves like a generation cycle, there is a need to identify the relevance of a measured number within the time scale, especially when regarding this measurements in the context of an ecological time frame.

Given the fact that the wealth of experimental ecotoxicity information exists for short-term standardised bioassays, and that for most chemicals this toxicity information is the only available, there is a long-lasting debate on the possibility to extrapolate chronic toxicity values from short-term toxicity data. Under the keyword acute to chronic ratio (ACR) several authors have generated experimental information for specific compounds using short-term and long-term exposure designs in distinct species (e.g. Morton et al., 1997). Others undertook to derive extrapolation factors from review of literature data for specific chemicals (e.g. Ford, 2001) or groups of chemicals (Länge et al., 1998, Roex et al., 2000) combining evidence from independent studies. Länge and co-workers (1998) e.g. compared ratios of EC50 values from acute studies to NOEC values from chronic toxicity studies for 71 substances. For that purpose, they drew their data for calculation from the factual database of the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) which puts special emphasis on the quality of the reviewed data with respect to the verification of toxicant concentrations in the studies to be included. Table 2 shows a descriptive statistics as an overview of their findings. Using the 90%ile as a descriptor of the distribution of ACR values for different groups of chemicals for which the analysis could be performed the ratio between reported acute and chronic toxicity values varied from a factor of 16 to almost 200. The median value of the acute to chronic ratio for all chemical is 8.6 which is in good agreement with an analysis based on species sensitivity distributions for 89 pairs of acute and chronic toxicity descriptions for information from 3 to 262 species (de Zwart, in press). Looking again at table 2, it seems striking that metals and specifically acting pesticides are the chemical classes with the higher ratios, though the wide distribution of data as seen in the minimum and maximum values also clearly warn of applying generalisations to individual cases. The notion that the mode of action rather than the structure of a particular chemical plays an important role in explaining different ACR values has also been brought forward by

Roex et al. (2000), who also show that the smallest variation in ACRs can be seen for nonpolar narcotic chemicals.

Table 2: Acute EC50 to chronic NOEC ratios (ACRs) for fish and daphnid toxicity data for groups of substances (taken from Länge et al., 1998).

substance group	no. of substances	acute EC50:chronic EC50 Min	50%-ile	Max	90%-ile
all chemicals	71	0.13	8.63	1290	72.9
pesticide a.i.	26	1.33	12.2	371	83.7
other organics	26	0.13	3.91	27.5	15.9
other organics, but at defined periods of exposure	19	1.25	3.60	28.3	24.5
metals and organo-metals	14	0.30	28.0	1290	192.0
other inorganics	7	2.92	8.39	69.3	20.1

Building acute to chronic toxicity ratios is, however, nothing more than trying to find rationales for extrapolation factors and thus dealing with the misery of regulatory biotesting using standardised protocols only, with arbitrarily chosen time periods for endpoint estimates. The alternative is available in form of functional descriptions of concentration-time relationships. For many years of pharmacological and toxicological research this is an issue of thought (Rand et al., 1995).

A canonic approach is to start with visualising concentration-time response surfaces. An illustrative example for comparing the metal toxicity against *Daphnia magna* clones has been provided by Barata et al. (1999). Figure 5 is taken from their work and illustrates that by simple transformation of the response scale according to a normal distribution, i.e. calculating probits a linear plane describes the experimental data in the three dimensional space already pretty well. Thus a multiple linear regression model of the form

$$E = a + b \ln(Conc) + c\ 1/\ln(T) \qquad \textbf{(Eq. 2)}$$

fits the data (with E, effect in probits; Conc, concentration of the toxicant; T, time; and a, b, and c, linear regression parameters). More sophisticated functional descriptions of concentration-response surfaces may be found in the pharmacological literature (e.g. Levasseur et al., 1998).

Simplifications of such an approach, undertake to reduce information to a two-dimensional plot in order to derive characteristic values such as median lethal/effective time or incipient lethal/effective concentration (Rand et al., 1995). As in concentration-response analysis

scale transformations are performed to allow simple regression techniques to estimate parameters of interest.

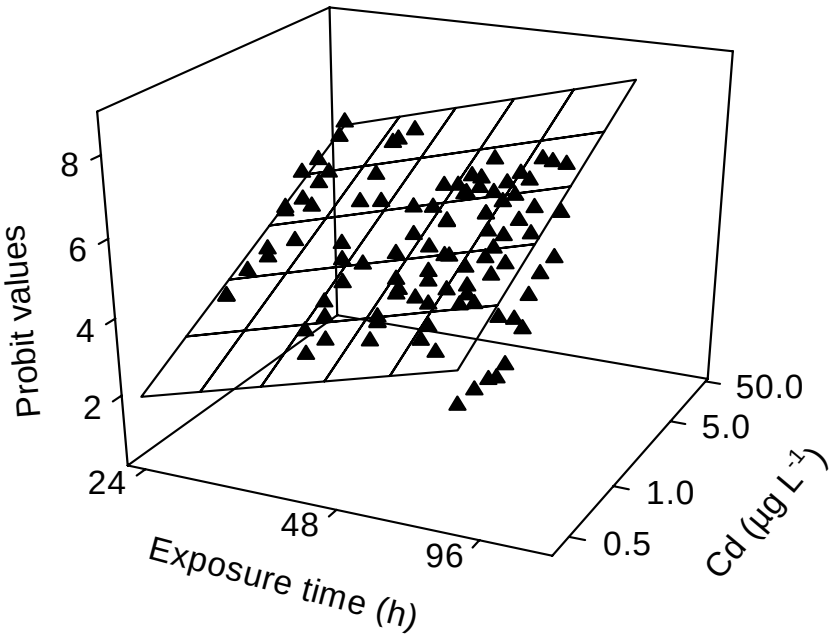


Figure 5: Concentration-time response surface for the effect of Cd on *Daphnia magna* immobilisation for 96 h checked in 12 hrs intervals (with kind permission from Barata et al., 1999).

An ecologically oriented theory that strives to describe toxic effects as process pertubations is the dynamic energy budget theory, which is formalised to the so-called DEBtox-model (Kooijman and Bedaux, 1996). On the basis of time series toxicity data for standard bioassays the DEBtox-model derives estimates for median effects like common concentration-response models. But in addition it generates no effect concentrations and time dependent toxicities. The modelling works modular assuming different kinetics and effect propagation concerning costs for growth, maintenance and reproduction for the different test organisms used in standard biotest protocols like fish or daphnids. The calculus of the DEBtox-software package relies on solving sets of differential equations for the kinetics and dynamics of the compounds.

Implicit to most time-response modelling efforts is the assumption that effect propagation following an exposure to a toxicant is a steady process. This is probably a reasonable assumption for many unspecifically acting compounds like solvents or other industrial chemicals. It has also been shown to hold for a group of organophosphorus pesticides, which irreversibly bind specifically with their metabolised oxon analogues to acetylcholinesterase (Legierse et al., 1999). For such cases a so-called critical target occupation model has been proposed that describes the concentration in an organism at the time of death as a product of the area under the time-target tissue concentration and a constant, which can be derived from bioconcentration models and standard toxicity estimates (Legierse et al., 1999).

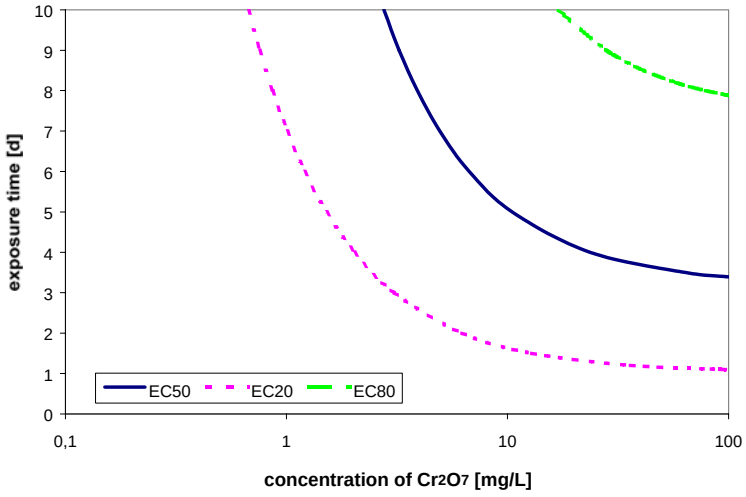


Figure 6: Time-response relationship for the effect of potassium dichromate on the frond growth of *Lemna minor* as calculated using the dynamic energy budget theory based DEBtox-model of Kooijman and Bedaux (1996). Depicted are lines of equi-effects on a concentra-tion-time scale (see chapter III and IV).

However, with specifically acting compounds one has to consider the possibility that primary interactions occur with processes at certain stages in the development of individuals and that such mechanisms of action will show a sensitive window in a life cycle. Examples for this are the sexual development of fish and its vulnerability to endocrine disruption (Segner et al., personal communication) or the action of antibiotics on microorganisms (Backhaus et al., 1997). An example of the latter which can be interpreted in terms of mode and mechanism of action is provided in figure 7. Gentamycin, an antibiotic known to specifically bind to bacterial 30S-RNA and thus interrupting protein biosyn-thesis, does not show any effect in water soluble con-centrations in standard lumi-nescence assays of 5-30 min exposure. However, if the test protocol is modified to allow a full cell cycle the antibiotic potency of the compounds is easily demon-strated.

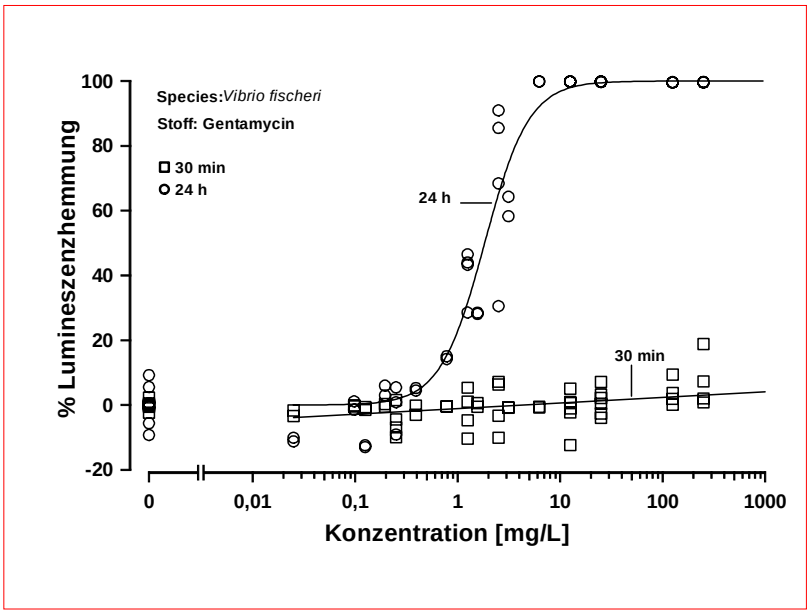


Figure 7: Effect of the antibiotic gentamycin on *Vibrio fischeri* after 30 min and 24 h of exposure (modified after Altenburger and Backhaus, 2000, see chapter IV).

4.3 Bioassay-directed fractionation and identification of toxicants

The function of this approach for an assessment of ecosystems is to identify compounds of toxicological potency in complex contaminated environmental samples and to establish a causal link between occurrence of contaminants and possible adverse effects on biota. Very

often in assessment of site-specific contaminations this knowledge cannot be adequately derived from existing emission information. In principle, one starts with defining the relevant toxic effect in the original sample. The original sample is than fractionated according to physico-chemical properties e.g. volatility, lipophilicity, or molecular size. The clue is to perform separation or clean-up in a way that allows subsequent testing of the remaining toxicity in the samples. The principle is illustrated in figure 8.

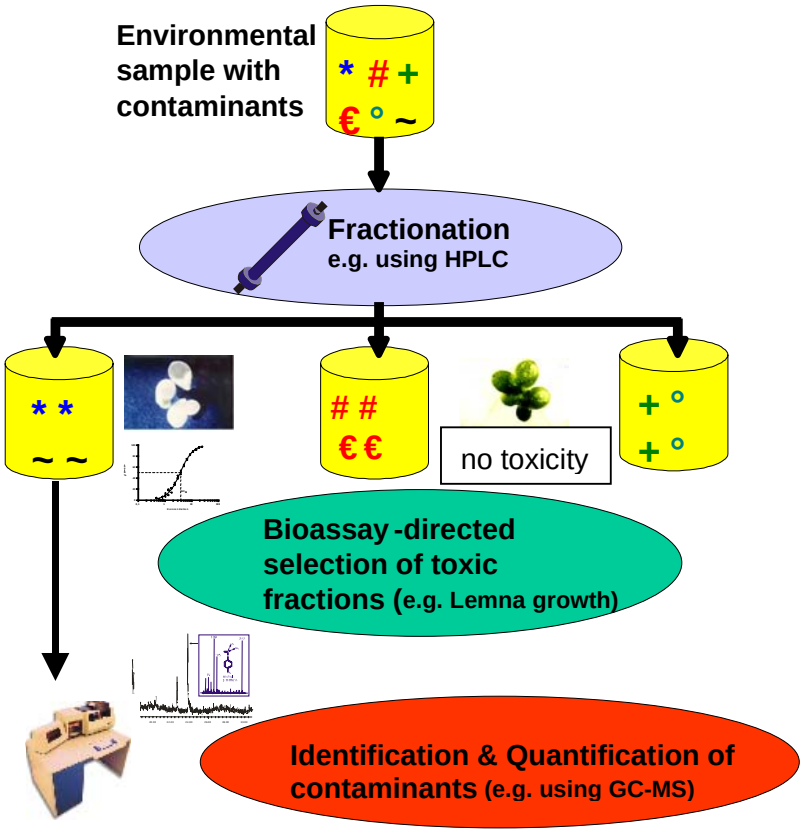


Figure 8: Illustration of the principle of bio-assay-directed fractionation and identification of toxicants in complex contaminated environmental samples (see chapter II).

For those fractions, that recover most of the toxicity of the original sample, this procedure may be continued in an iterative process, employing different separation principles. This process is continued until a chemical analysis concerning the elucidation of structures and their amounts due to sufficient clean-up can be performed. Chemical analysis is thus restricted to those fractions that retain toxicity. Also, it is no longer guesswork as to which identification technique might be adequate, due to the information gained from the fractionation techniques employed.

As one example of this approach we consider a study by Brack and co-workers (Brack et al., 1999) who investigated a highly contaminated sediment in the riverine Spittelwasser, Germany, which flows into the river Mulde, a tributary to the Elbe stream. The Spittelwasser drains the areas of Bitterfeld and Wolfen, two of the major chemical production sites in Europe for over a hundred years. After political change and close-down of most production facilities, this riverine is to be remediated now. Though information on various contaminants exist, knowledge of the priority toxicants, however, is lacking. As production ceased the water body is not any longer considered a priority problem, in contrast to the still heavily contaminated sediment.

To elucidate the composition of contaminants with potential ecotoxic effects, sediment samples from the creek were taken and Soxhlet-extracted with acetone. Such an extraction procedure allows to recover organic compounds of medium polarity to high lipophilicity. Metals and / or highly polar organic contaminants, however, will be lost. These extracts were then fractionated and biotested in a stepwise procedure till components could be identified using gas chromatography with mass selective detection (GC/MSD). Three biotests representing different life forms and types of response were used as effect detectors: namely cellular reproduction of the unicellular green algae *Scenedesmus vacuolatus*, mobility of the water flea *Daphnia magna* and bioluminescence of the bacterium *Vibrio fischeri*. The so-called confirmation step comprises of testing pure compounds that have been analytically identified in the effective subfractions and comparing effect concentrations determined for the pure compounds with the effect dilution for the fractions for which compound quantities can be estimated. Besides well-known and expectable toxicants, like organotin compounds and several polyaromatic hydrocarbons (PAHs) this study revealed effective concentrations of active ingredients of pesticides like prometryn (used in herbicides) and methyl parathion (used in insecticides) as well as a completely unexpected toxicant, namely N-phenyl-ß-naphthalene amine. While a compound like parathion would not have been expected due to its rapid degradability in this system, the high phytotoxicity of N-phenyl-ß-naphthalene amine was first identified in this study. Obviously, all bioassays employed detected different toxicants, thus proving, that the use of biotest batteries is necessary whenever there is no pre-defined focus for a specific toxic effect. Thus any remedial action considered, could now use criteria to assess biological efficiency of remediation activities in addition to purely chemically defined ones.

A second example where a defined effect quality was considered is given by Purdom and co-workers, who undertook an elegant work to identify the causes of the previously reported estrogenic potency of effluents from sewage-treatment plants in British rivers (Purdom et al., 1994). Sewage treatment plants, coping with industrial and domestic waste release highly complex effluents. Particularly, the non-ionic surfactant group of alkylphenols from household detergents are suspected to be responsible for the estrogenic potency of effluents, due to *in vitro* evidence. Fractionation of crude effluents of several sewage treatment plants into sub-samples containing volatiles, particulates and dissolved compounds in a first separation step using an in vitro yeast-based screen for estrogenic activity rendered the dissolved phase as the only fraction containing any bioactivity. Three further fractionation steps, separating compounds according to lipophilicity of components on C18-solid phase extraction cartridges and subsequently on C18-HPLC columns left but a few active fractions. GC-MS analysis of these purified fractions identified estrone, 17ß-estradiol and 17α-ethynylestradiol as the principal components. While the former two are supposedly of natural, human origin the latter compound is the main estrogenic component of the combined oral contraceptive pill. In a supplementary paper, the allocated effect quality of estrogenic responses was further validated for 17ß-estradiol, estrone and an octylphenol (Routledge et al., 1998). In *in vivo* tank trial experiments, adult male rainbow trout (*Oncorhynchus mykiss)* and adult raoch (*Rutilus rutilus*) were exposed for 3 weeks to environmentally relevant concentrations of these compounds and the vitellogenin (VTG) content of blood samples was determined. The

stimulation of the production of the female egg yolk protein VTG was used as a biomarker of response indicating estrogenic contamination. All compounds investigated elucidated similar responses, however the natural steroidal estrogens were 3 orders of magnitudes more potent as compared to octylphenol and furthermore they showed potencies at environmentally relevant concentrations.

Major limitations for using results from bioassay-directed fractionation to predict ecosystem effects result from the high demand on testing capacity and the lack of bioavailability information. The first point is a technical aspect namely the limited use of laborious techniques to determine e.g. long-term, chronic effects which in turn preselect effect parameters and considered targets at risk. In this respect, intelligent experimentation techniques such as molecular biomarkers or high-troughput devices are needed. The second issue, namely the assessment of bioavailability of compounds in the ecosystem context can only be addressed when complementing studies of site-specific toxicant identification, with investigations to determine the bioavailability *in situ*. An interesting approach to not only determine the bioavailability of organic pollutants in aquatic systems but additionally verify their toxic potentials has been provided by a combination of using semipermeable membrane devices as bio-mimetic passive sampler and employing bioassays on derived extracts from them (Sabliunas, 1999).

4.4 Effect analysis

The purposes of studies that focus on effect analysis in order to gain understanding of the mode of action of substances are in this context:

- to understand the relation between a substance and biological responses in order to identify sensitive taxa and processes within ecosystems;
- to clarify the scope for inference of effect assessments between different species;
- to assess the relevance of specific effects for ecosystem well-being and predict whether found environmental concentrations of contaminants may be linked to adverse effects.

Much of the variation seen in different organisms in response to toxicants has been attributed to the mode of action of chemicals (Vaal et al., 1997a). Moreover, while non-reactive organic chemicals, that act via unspecific so-called narcotic action reveal relatively small sensitivity distributions of acute toxicity data and commonly are straightforward to model regarding their acute to chronic toxicity relationships, the contrary seems true for reactive or specifically acting compounds (Figure 9) (Vaal et al., 1997b).

For specifically acting compounds the interference with taxa-specific processes or targets is of course the background for utilisation of compounds as active ingredients of drugs like antibiotics, herbicides, etc.. A current review of the understanding of unspecific toxicity can be found in Caisukant, Yu and Connell (1999), reviews of specifically acting compounds may be found in text books on phytopharmacology and human and veterinary drugs.

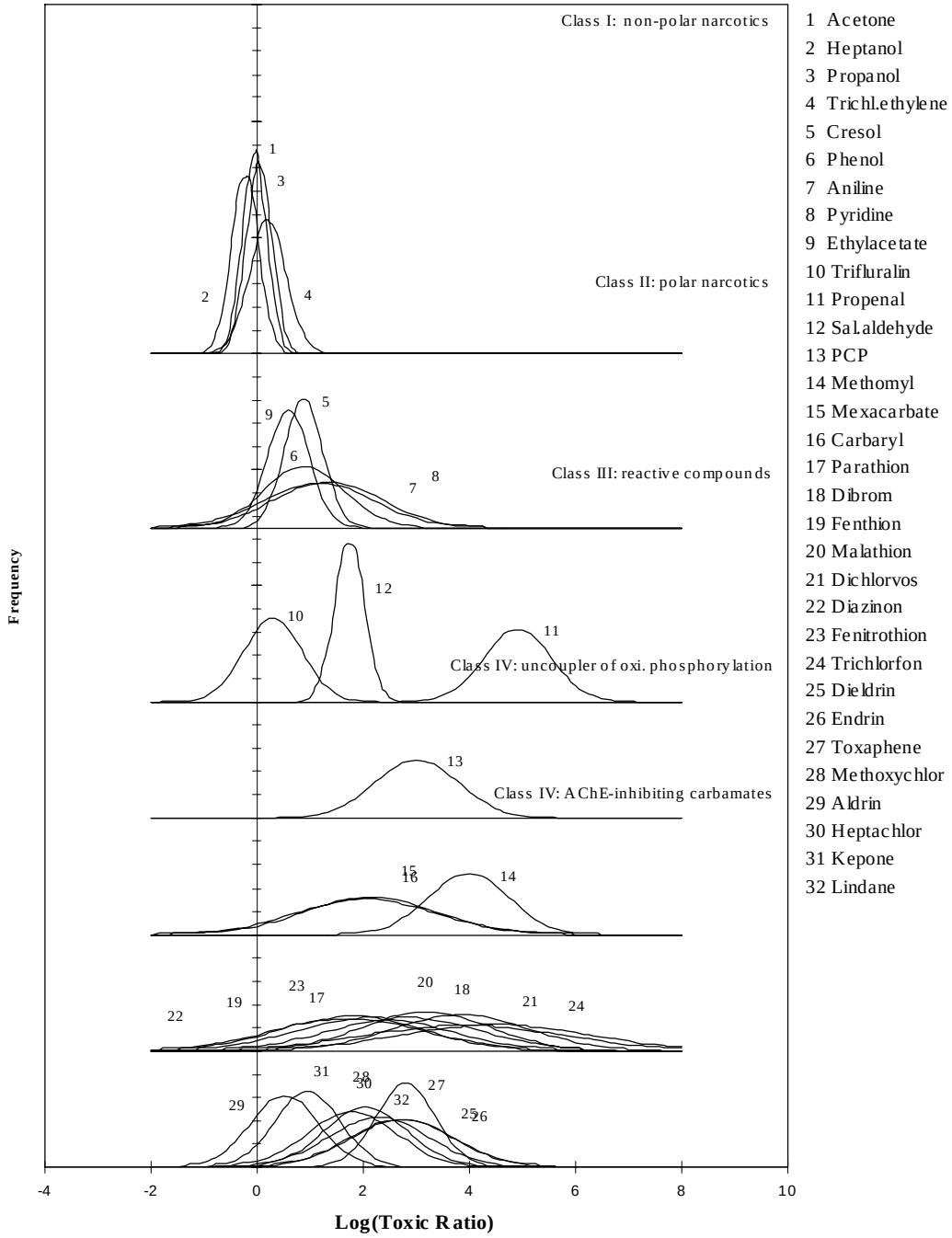


Figure 9: Sensitivity distribution of acute toxicity data for compounds with known mdes of action (with kind permission from Vaal et al., 1997b).

For most chemicals in the environment, however, the mode or even mechanism of action is unknown. When we aim to protect ecosystems, we have to acknowledge their property to consist of assemblies of various life forms and strategies. Commonly, the way to handle the resulting information gap on the toxicity potential for all organisms present in a specific ecosystem, biotest batteries consisting of selected species are constructed. These can be built on rationales like representing (i) different trophic levels of a food chain, (ii) various levels of biological complexity, (ii) different life strategies, or (iv) endangered species. Reviews on the ecotoxicity potential of specific compounds like Chlorate (Van Wijk and Hutchinson, 1995) or 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (Boening, 1998) illustrate this thinking to identify most sensitive taxa. The challenge is to obtain information for specifically

sensitive organisms as in the example of chlorate the toxicity to aquatic organisms and ecosystems is relatively low for most species with values in the higher mg/L-range but very high for several species of marine macroalgae of the genus *Fucus* and for micro-phytoplankton communities with values in the µg/L-range (Van Wijk and Hutchinson, 1995). More often, the available information on the toxicity of chemicals towards different species is strongly biased towards species of particular economic impact like fish or bees and assessment efforts are thus hampered (Boening, 1998).

A different perspective comes from approaches assembling bioassays that represent different physiological competence and thus interaction potentials or that utilise batteries of biochemical and subcellular assays which may go as molecular as specific receptor-binding assays. The former approach is typically realised by constructing multi-parameter test-systems for single species like the observation of fish acute toxicity syndromes (McKim et al., 1987), the differentiation of lethal effects on egg and adult stages and non-lethal effects on food acquisition and production rates over time in *Daphnia* females (Barata and Baird, 2000), or the observation of different structural and functional parameters in the synchronised life cycle of unicellular green alga (Grimme et al., 1993). The latter strategy may combine in vitro assays that reflect different modes of action like the inhibition of acetylcholinesterase through organophosphorous compounds or the decoupling of oxidative phosphorylation through specific phenolic compounds (Wenzel et al., 1997). These approaches may correctly assess or predict the quality of the toxic potential of specific chemicals, however care must be taken regarding the quantification of the concentration-response relationships as *in vitro* tests are often less sensitive compared to organismic responses (Wenzel et al., 1997). A second problem in the prediction of the toxicity potential for ecosystems from a mode of action approach is the discrepancy of the recognition of a limited number of 7-10 modes of actions as discussed in the ecotoxicological literature (Nendza and Müller, 2000; Schüürmann, 1998, Wenzel at al., 1997), and the knowledge and utilisation of many more specific targets in drug application. Thus, Faust et al. (2000) could extract 40 different specific mechanisms of actions for herbicides and Backhaus et al. (2000) 32 mechanisms of action for antibiotics using textbook knowledge only. Also, the young history of ecotoxicology is a history of surprises regarding the discovery of new effect qualities like the current debate on endocrine disruption shows (Matthiessen, 2000).

An example for the kind of surprises that we face and that most often will not reach newspaper headlines derives from a bioassay-directed fractionantion of a contaminated sediment in the Bitterfeld area (see chapter II). The compound N-phenyl-β-naphthalene amine used as a rubber antioxidant in industrial production has been found in the analysed sediments in amounts of about 15 mg/kg sediment and was identified of so far unreported high phytotoxicity (Brack et al. 1999). Subsequent biological investigations are summarised in figure 10. In the water soluble range short-term exposure (up to 48 hrs) against N-phenyl-β-naphthalene did not elucidate effects in bacteria or daphnia. This is consistent with chemical fact sheets available for the substance including fish data. In the chronic one-generation algal test that we employed, however, concentrations of a few µg/L proved to be effective. This efficiency compares to specifically acting photosystem II inhibitors or highly

phytotoxic compounds like copper. The estimated minimum toxicity due to an unspecific narcotic mode of action (see 4.6 and chapter V) would be in the range of 10 mg/L. We therefore investigated several plant processes as possible specific targets of N-phenyl-β-naphthalene amine action. Germination of water cress, heterotrophic growth of pollen tubes of tobacco and protein synthesis in peas did not show significant disturbances up to an exposure of 10 mg/L. Only non-photochemical quencing of variable fluorescence in algae showed concentration-response relationships comparable to the inhibition of algal reproduction. However, it was observed that the responses were orders of magnitude lower when exposure lasted for shorter time periods. Cellular growth analysis confirmed the importance of a prolonged exposure for elucidation of high toxicity. Comparison of the responses in the analysis of a puls amplitude modulation induced fluorescence patterns of N-phenyl-β-naphthalene amine with atrazine and CCCP revealed that the former compounds is somewhat similar to CCCP and rather distinctly different from atrazine (data not shown). Thus the current working hypothesis on the phytotoxic action of N-phenyl-β-naphthalene amine is, that this compound is slowly accumulated at the membrane target sites where it subsequently efficiently destroys the proton gradient over the cloroplast membrane, thereby depriving affected organisms of energy generation.

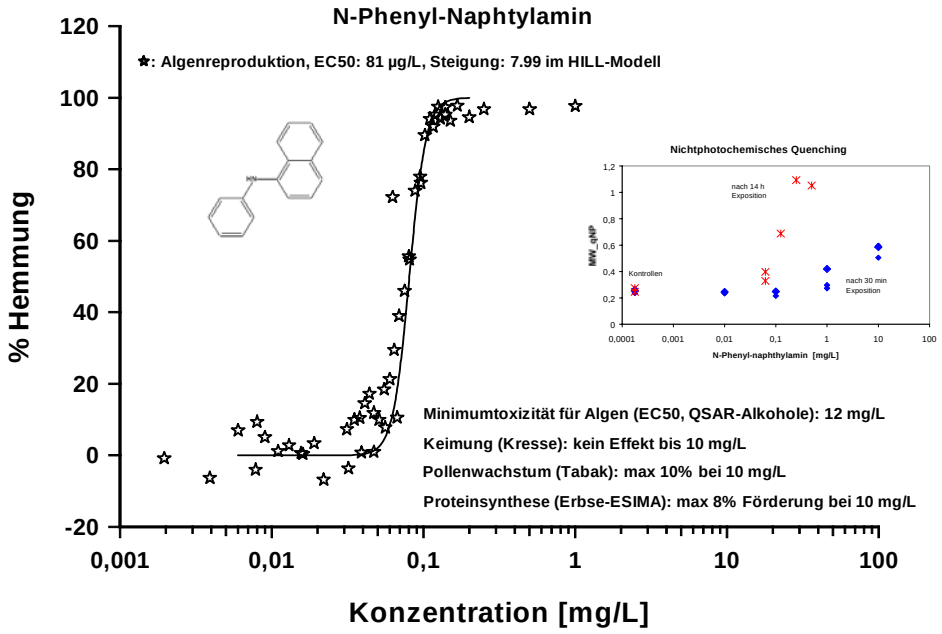


Figure 10: Effect profile for N-phenyl-β-naphthalene amine.

A serious problem regarding the predictive scope for mode of action information apart from the concentration argument derives form the necessity to understand effect propagation from a molecular level of biosystem pollutant interaction to interference with population and community relevant parameters. The question when we do understand the mode of action of a specific chemical is, how does it feed through the different levels of biological organisation towards a response that is to be seen on the population level. The linkage of parameters relevant at the population level like behaviour, growth, reproduction and mortality to physiological observations is often tried using energy budget considerations as observable in

short-term experimentation (Kooijman and Bedaux, 1996, Barata and, Baird 2000, Knops et al., 2001). An example for this is provided in figure 11, where the results of an exposure of *Daphnia magna* females for eight days against a cationic surfactant (CTAB) and the metals copper and cadmium on scope for growth, dry weight increase and egg production are displayed (Knops et al., 2001). While scope for growth calculations based on oxygen consumption and food (algae) intake measurements showed a good correlation to achieved dry weight increases, the parameter most relevant for population performance namely egg production shows a compound specific response. This demonstrates that there are no simple deterministic links between the parameters but that resource allocation is flexible and effect propagation a process in itself.

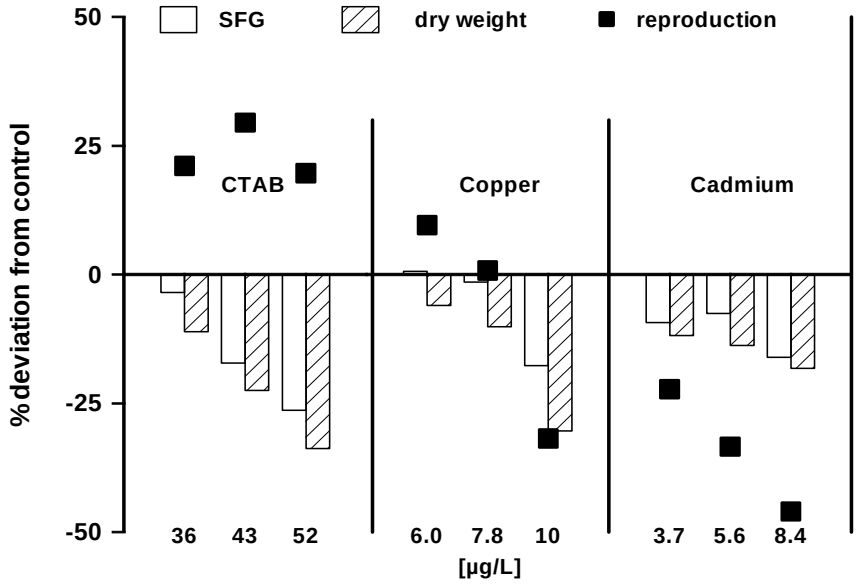


Figure 11: Effect propagation from physiological levels to population relevant responses (adapted from Knops et al., 2001). Scope for growth (SFG) was modelled on the basis of oxygen consumption and food intake measures (see chapter VII).

Typically, the effect assessment of compounds or contaminated environmental samples is based on single species studies that are performed under physiologically optimised conditions. This is done for the good reason, that a maximised signal to noise ratio is appropriate in effect quantification as discussed in various monographs on biotest development (Wells et al., 1998, Steinhäuser and Hansen, 1992). Regarding the prediction of effects based on these type of data, one has to consider that organisms outdoor have to cope with various environmental constraints, that may affect the sensitivity against exposure with pollutants. Examples have been provided demonstrating that the interaction due to density effects in a Daphnia population may alter responses to chemical stress (Goser, 1997). It has also been shown that interaction of environmental factors and chemical stress might affect responses of organisms. Thus, UV exposure and food ration increased the sensitivity of the amphipod *Paramoera walkeri* against copper exposure (Liess et al., 2001).

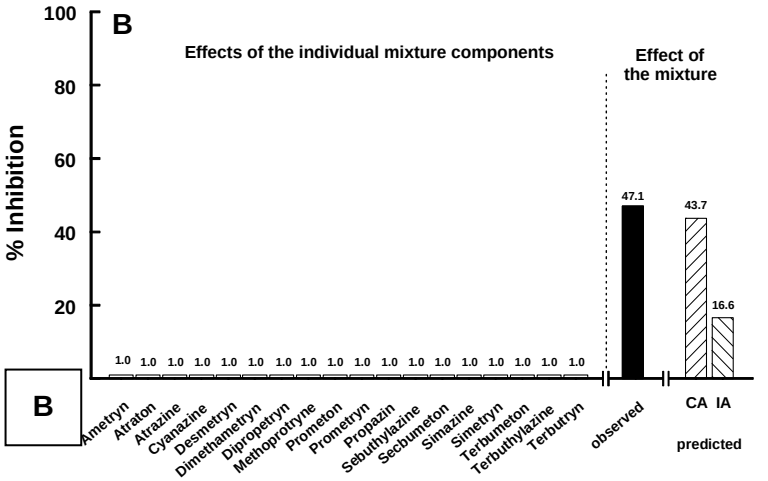
4.5 Combined effect analysis

Contamination of the environment is rarely a matter of single chemicals but rather of mixtures of components. This raises the question of the occurrence and relevance of combined effects for individual species as well as for communities or ecosystems. A differentiated methodology based on two pharmacologically founded concepts has been developed that comprise not only the possibility of developing rational and experimentally accessible approaches to this field but that also offers the opportunity to overcome the terminological confusion close to anarchy which still prevails in the field (Altenburger et al., 1993, Greco et al., 1995, Kortenkamp and Altenburger, 1999).

There is a vast body of literature studying how binary or multiple mixtures affect various biological responses usually observed in individuals of single species (Greco et al. 1995, Altenburger et al., 1993, Greco et al., 1995, Kortenkamp and Altenburger, 1999). The discussion employs terms like synergism or antagonism to qualify the observed effects. Synergism or antagonism are commonly taken to mean that the observed effect of a mixture was more or less than what had been expected. Thus the central question for any assessment of the effects provoked by mixtures is: What is a reasonable expectation for combination effects (Berenbaum, 1981, 1985, 1989)? Very early in the 20th century two different concepts that can be based on pharmacodynamic assumptions namely Concentration Addition and Independent Action were formulated that allow to calculate expectable combined effects on the basis of information on the efficacy of the single components (Berenbaum, 1981, Greco et al., 1995). There are main differences of this concepts: Concentration Addition is based on the idea that one substance may act as an equitoxic dilution of another or in pharmacodynamic thinking calculates combined effects for substances that have a similar mechanism of action. - Independent Action in contrast, regards effects of components as statistically independent and is thus thought to be valid for situations were the mixture components show dissimilar mechanisms of action (Grimme et al., 1996).

When moving from a pharmacodynamic level of molecular interaction to the assessment of mixture toxicities, a first question is whether simple ideas about combined effects at the level of molecular receptors translate into meaningful expectations at the level of intact organisms. Using two photosynthesis inhibitors with a known identical molecular binding site, Altenburger et al. (1990) studied mixture responses at different levels of plant responses employing Hill reaction measurements to quantify interaction with photosynthetic electron transport in isolated chloroplasts, photosynthetic oxygen production of algae after 15 minutes of exposure, cell volume growth performance after one growth phase and finally reproductive success after one generation. They were able to show that indeed concentration addition is a suitable concept for assessing combined effects on different levels of biological responses for this case of compound mixture with an identical mode of action. In subsequent studies the same group demonstrated for over a hundred different binary mixtures of pesticides and surfactants using an algae reproduction assay that indeed both concepts Concentration Addition and Independent Action provided quantitatively reasonable reference values for

combined effect assessments (Faust et al., 1994, Altenburger et al., 1996). The case of multiple mixtures of compounds with unspecific modes of action like industrial chemicals such as solvents has been addressed in a series of studies by Köneman (1980, 1981), and by Hermens and coworkers (1982, 1984, 1985) using fish, daphnia and bacterial toxicity parameters. Even for cases where the concentrations of the individual chemicals where as low as 0.02 of their individual EC50 values significant combined effects where observable and close to what would be expected from concentration additive behaviour. The thus anticipated dispute as to whether concentration addition might be an universal model for higher levels of biological responses (Berenbaum, 1985, Pöch, 1993) became experimentally addressed in a series of papers by Grimme and colleagues (1996). They deliberately combined multiple target specific mixtures of drugs with well known molecular modes and mechanisms of action and investigated them in a bacterial and an algae toxicity assay (Altenburger et al., 2000, Backhaus et al., 2000, Faust et al., 2000, see chapter V). Figure 12 shows one of their striking findings, namely that indeed the knowledge about the mechanism of action provides a means to accurately calculate mixture toxicities for similarly or dissimilarly acting compounds (Faust et al., 2000).



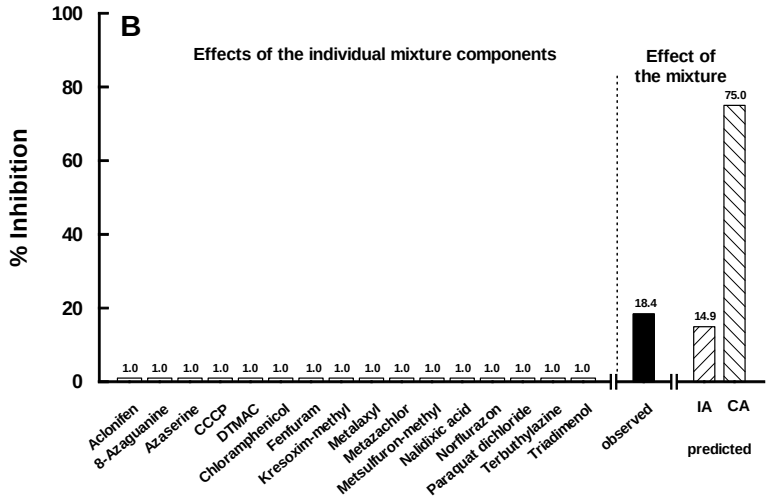


Figure 12: The mechanism of action provides a means to accurately calculate mixture toxicities for (A) similarly or (B) dissimilarly acting compounds (adapted from Grimme et al., 1998, see chapter X).

The question whether the tools and understandings developed and validated in single species investigations are transferable to higher biological hierarchies has been addressed by Blanck and coworkers. In a first study an investigation on the combined effect of tri-n-butyl-tin and diuron on marine periphyton communities detected as pollution-induced community tolerance (PICT) was made (Molander et al., 1992). The authors were indeed able to detect not only single compound activities but also combination effects. From response surface modelling they concluded that for the investigated mixture

PICT-responses could be interpreted as occurrence of co-tolerance. Subsequent work performed with marine periphyton and epipsamon communities (communities, grown on sand) showed that while responses show higher variability as compared to lab-based single species algal toxicity testing, the type of mixture toxicities to be predicted and observed might be very similar to what has been described above (Vighi et al., accepted).

The relevance of these recent advances in the understanding of mixture toxicity with regard to assessment schemes based on PNEC values for single compounds is currently discussed (Faust et al., 2001, Faust et al., submitted, Walter et al., accepted, see chapter VIII). Of course not all mixtures of contaminants to be found in the environment just behave as expected on the basis of a simplistic pharmacological reasoning. There are well documented cases of unexpected high combined effects on population and organismic levels of responses (Johnston et al., 1994, Babu et al., 2001). And there are of course investigations as to the mechanistic understanding of such interactions, which either focus on processes related to energy transducing membranes (Escher et al., 2001, Schweigert et al., 2000, 2001) or on interactions via altered internal contaminant concentration due to interference with biotransfomation enzymes (Johnston et al., 1994).

With regard to predictions of mixture toxicities to ecosystems apart from problems of correlated responses like co-tolerance occurrence or interference from indirect effects when considering mixture with dissimilarly acting components, the definition of the type and ratio of mixtures to be assessed in terms of what is the actual exposure situation pose major challenges for the future.

4.6 Quantitative structure-activity relationships (QSAR)

As illustrated above there is the tremendous lack of even the most basic experimental data in ecotoxicology for most chemicals in everyday use. Considering a number like the about 100000 chemicals which are listed in the European Inventory of Existing Commercial Chemical Substances (EINECS), it is apparent that neither resources nor the will may be allocated to change this picture in due course. The study of quantitative structure activity relationships offers an approach to tackle the problem of lacking experimental data on biological effects. The scope for investigations of structure activity relationships is to provide estimates of compound intrinsic biological activity properties in a systematic manner, i.e. to provide a generic toxicity profile. The basic principle lies in the comparison of several compounds of a similar structure (congenerity criteria) with respect to a defined biological activity at a fixed effect level. Various structural parameters may than be used to describe and analyse the observed effectiveness of the components of training and validation sets. Employing statistical methods e.g. regression techniques, predictive tools applicable for untested compounds can be derived. Good accounts of the principles of this methodology as utilised in ecotoxicology may be found in Nendza and Hermens (1995) and Schüürmann (1998). This approach has played a major role in reviving the concept of narcotic action of substances and QSARs derived from the correlating toxicities of non-polar, non-reactive

organic compounds with compounds lipophilicity i.e. logK_{ow} allow to estimate a baseline toxicity for almost any given organic chemical (Lipnick, 1989). Such values for an expectable minimum toxicity can be used immediately as a prediction in effect assessment, but may also serve as a reference to judge about the existence of more specific interactions with biological systems.

Unlike its application in pharmacological studies where the preselection of an effect of interest is often highly appropriate, QSAR approaches to be used in ecotoxicology, have to cope with various possibilities of interactions of chemicals with biological systems. Thus the reflection of the compound selection is a crucial issue. Pioneering work of Verhaar, van Leeuwen and Hermens (1992) proposed a scheme based on earlier work to classify various organic chemicals into one of the following four classes: inert chemicals, less inert chemicals, reactive chemicals and specifically acting chemicals. Applying this scheme to 2000 chemicals labelled by the OECD as so-called high production volume chemicals (HPVCs) allows consideration of already 44% of these chemicals (Bol et al., 1993). For the compounds classified as inerts QSAR equations to predict the short-term median aquatic toxicity values EC50 for fish, daphnia and algae based on a narcotic mode of action were calculated using the compounds octanol/water partioning coefficient logK_{OW} as sole structure parameter (Verhaar et al., 1992). For the other groups that are expected to show somewhat higher toxicities due to interactions other than mere unspecific membrane disturbance group specific empirical factors multiplied with the baseline toxicity value were proposed and used (Bol et al., 1993, Verhaar et al., 1992). This work became extended using various QSAR estimates for the toxicity for other organisms and endpoints like NOECs and deriving quality criteria for aquatic ecosystems based on species sensitivity distribution functions that were generated from the 19 estimated toxicity values (van Leeuwen et al., 1992). For risk assessment and management purposes this type of work allows coping with many chemicals that have yet not been assessed by regulators (Verhaar et al., 1992, Bol et al., 1993). However, it requires that hazard predictions based on suspicion rather than on numbers derived from base set routine test data become acceptable to risk regulators.

Inert chemicals evoking baseline toxicity, however, are to be among the least toxic substances, while toxicities higher for up to several orders of magnitude due to reactive or specifically acting compounds may be more crucial for ecosystems. Thus the challenge to discriminate further modes of action rather than just narcosis and allocate adequate QSARs has been taken up by various groups (Niculescu et al., 2000, Vaal et al., 2000, Marchini et al., 1999, Kapur et al., 2000, Parkerton and Konkel, 2000, Escher et al., 1999) of which two will be highlighted for illustration. A recent study by Basak and coworkers (Basak et al., 1998) employed molecular similarity, neural networks and discriminant analysis to assign the mode of action out of seven different types using acute fish toxicity data. For a set of 283 chemicals for which information as to the mode of action was available a correct assignment of 65 to 95% of these chemicals was possible. Similar outcomes were achieved when allocating 115 test chemicals to nine modes of action using quantum chemical descriptors and principal component analysis (Nendza and Müller, 2000).

Other tasks of QSAR studies regarding the predictive scope of this approach for ecosystems are oriented towards understanding the structural determinants of compounds to elicit effects of ecotoxic relevance. The recently heavily debated potency of various structurally unrelated pollutants to infere with the endocrine systems of heterotrophic organisms (Matthiesen, 2000, Jobling et al., 1995, Tyler et al., 1998) has launched activities to understand the structural determinants of estrogen receptor binding (e.g. Xing et al., 1999 Tong et al., 1998). For a group of nitroaromatic compounds that QSAR based effect assessment as proposed by the US-EPA would consider as merely narcotically acting and thus predicting a baseline toxicity Schmitt et al. (2000) showed for algal reproduction toxicity data that these compounds are in general more toxic than nonpolar narcotics. Moreover, additional inclusion of quantum chemical electronic parameters like the energy of the lowest unoccupied orbital (E_{LUMO}) gained a consistent quantitative structure activity relationship for all nitroaromatic compounds. In turn, employment of these structural parameters allowed to suggest additional modes of action in the organisms such as oxidative stress evoked from redox cycling of some of the compounds and toxicity from metabolites due to biotransformation (figure 13).

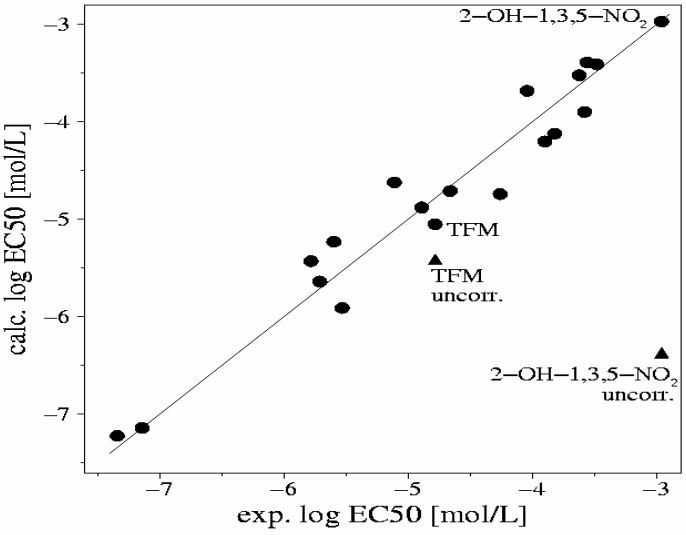


Figure 13: Calculated versus experimental log EC_{50} for inhibition of alga reproduction by nitrobenzenes using a three-variable regression model of the form log EC_{50} = -0.55logD_{OW} + 1.69E_{LUMO} - 34.3 $q_{nitro\text{-}N}$ + 18.4 (from Schmitt et al., 2000). For TFM and picric acid the triangles indicate their predicted log EC_{50} when using logK_{OW} instead of logD_{OW}. Abbr. $D_{OW,}$ partition coefficient between octanol and water for the un-ionised species; E_{LUMO}, energy of the lowest unoccupied orbital; $q_{nitro\text{-}N}$, net atomic charge at the nitro nitrogen (see chapter VI).

Finally, QSAR studies have occasionally treated the problem of chemical mixtures. The study of the mixtures of contaminants has been addressed using QSAR techniques to predict altered compound properties like modified solubilities and combined effects. Particularly, QSAR approaches have been used in the analysis of joint toxicity of chemicals to provide evidence for similar mode of action and thus concentration-additive mixture toxicity (see 4.5); to predict effect concentrations of untested components; to describe specific mixture effects deviating from expected responses; to discriminate between congeneric structures of dissimilar reactivity; to model exposure concentrations; and to derive mixture properties for prediction of joint toxicity (Altenburger et al., submitted).

Whatever the successes may be in filling data gaps by using quantitative structure activities relationships, the major drawback regarding inference from this approach to ecosystems is the necessary preselection of effect parameters and targets at risk. This limitation has at least two aspects: firstly, the understanding of toxic action of pollutants in biosystems as a process involving pharmacokinetics and -dynamics as well as effect translation from mechanisms of interaction to modes of toxicity is ignored. Secondly, as QSAR studies perform and require comparisons of many compounds though in principle they may consider any type of effect in practise this places high demand on testing capacity and limits use of laborius techniques. One therefore, will hardly find data allowing a QSAR analysis say for chronic toxicities not to speak of population or community level effects.

5 Scope for predictions

The methodologies introduced in the above chapters all have their specific drawbacks concerning the scope to assess and predict ecosystem responses. The validity of quantitative hazard estimations of compounds or effluents based on laboratory investigations using single species is easily challenged on reasons of site-specific bioavailability of pollutants, intra- and interspecific variability, altered responses of organisms in the community context, relevance of environmental factors like feeding status influencing population sensitivities, and undetected indirect effects. Various approaches address these problems in extrapolating from isolated single species tests to top higher levels of complexity. In the following we will briefly consider:

- *in situ*-toxicity testing,
- species sensitivity distributions,
- comparative studies using laboratory assays and micro- and mesocosm studies, and
- ecological considerations.

5. 1 In situ-toxicity testing

A straightforward methodology to examine the influence of site-specific environmental milieu factors on estimations of effect concentrations is to perform toxicity testing *in situ*. Representing site-specific milieu factors in toxicity testing may mean including specific sediments or water bodies in static systems like tanks, ponds or ditches, flow-through systems like artificial stream experimentation (Debus et al.,. 1996, Drent and Kersting, 1993, Girling et al., 2000, Rand and Clark, 2000), using bypass channels (Liess and Schulz, 1999) or caging of fish or macroinvertebrates directly on site (DelaTorre et al., 2000, Pereira et al., 1999, Ireland et al., 1996, Pyle et al., 2001). A review on various existing designs for these kind of outdoor studies can be found in Caquet et al., (2000). Numerous parameters can thus be monitored and a major challenge becomes the task to reduce data to meaningful information (Girling et al., 2000, van Wijngaarden et al., 1996).

The environmental impacts of acid mine drainage (AMD) was investigated in a case study at the Puckett's Creek watershed in Virginia, USA using benthic macroinvertebrate sampling, *in situ*-toxicity testing with Asian clams (*Corbicula fluminea*), water column toxicity testing with the cladoceran *Cerodaphnia dubia* and sediment toxicity testing with the cladoceran *Daphnia magna* and the midge larvae of *Chironomus tentans* (Soucek et al., 2000). Comparison of the different biological parameters investigated for 21 different sampling sites categorised for different AMD impacts revealed a fairly consistent pattern of biological responses for the sites exposed to acidic and neutral mining drainage. The water column testing for short-term survival of *Cerodaphnia dubia* not only proved to be very sensitive in terms of distinction between different sites but showed to correlate significantly with different indices describing the sampled benthic microinvertebrate community (r - values ranging from 0.49 to 0.81) (Soucek et al., 2000). The testing with clams (*Corbicula fluminea*) showed an almost identical response pattern regarding survival after 31 days of *in situ*-exposure compared to *Cerodaphnia dubia.* A similar study performed on a long-abandoned mining site located in south-eastern Portugal compared laboratory test results for water column and solid phase samples with caged *in situ*-testing using the cladocerans *Cerodaphnia dubia* and *Daphnia magna* (Pereira et al., 1999). Apart from a general good agreement in the observable short-term toxicities for 8 different sites at 4 sampling periods covering all seasons of the year, the mortality tended to be slightly higher for the bioassays, performed *in situ* and more similar to the solid phase tests (Pereira et al., 1999).

In a study with pyridaben, an active ingredient of a pesticide used as insecticide and acaricide, Rand and Clark (2000a, b) compared short-term toxicity findings for bluegill sunfish (*Lepomis macrochirus*) and mysids (*Mysidopsis bahia*) from laboratory studies using standard protocols with outdoor tank studies using natural photoperiod, single-pulse exposure and tanks filled with a defined sediment and specified water. They found that estimated LC50 values after 96h exposure increased from laboratory conditions to outdoor studies for both organisms by about 1.5 orders of magnitude (Rand and Clark, 2000b). This significant decrease in toxicity is not too surprising considering that the actual concentration of pyridaben was halved in the tank studies about 8 hrs after application and regarding the low water solubility of about 12 µg/L as well as the high lipophilicity as characterised by an octanol water partition coefficient log K_{OW} of about 6.4. The environmental behaviour of this compound would thus be expected to strongly favour partioning from the water column and sorption onto organic particles and sediment (Rand and Clark 2000a). The degree to which these factors alter observable toxic effects and possibly compound assessment, however, will always be site-specific.

Liess and Schulz (1999) tried to link rainfall-induced surface runoff from arable land contaminated with several insecticides and subsequent exposure of the macroinvertebrate community in adjacent streams with the abundance of several macroinvertebrate species. They employed a runoff-triggered sampler to follow insecticide contamination after rainfall-induced runoff and were able to quantify parathion and fenvalerate exposure via the water column and suspended particles after several such events. In order to distinguish between stress factors accompanying runoff events in the stream like increase in current velocity and

insecticide exposure, the authors used parallel bypass microcosms to isolate effects of contamination on survival and emergence of trichoptera larvae of *Limnephilus lunatus* and on survival of the amphipod *Gammerus pulex*. For a rainfall event where 6 µg/L of parathion could be detected in the swelling stream water for about one hour, significant decreases of the abundance of the populations of both organisms could be detected. These reactions are well in accordance with effect concentration data from several laboratory assays describing the short-term toxicity of parathion in *Gammerus* spp. (EPA-databank ECOTOX, http://www.epa.gov/ecotox/). Surprisingly, a short exposure period of only one hour suffices to reproduce these effects so exactly, that one might interpret the pharmacology of parathion on the basis of these findings as being very fast in uptake and provocation of mortality.

Maltby et al. (2000) investigated the biological impact of a point source discharge downstream a bleaching work. Whole effluent testing in the laboratory predicted an acute toxicity for neonates of *Daphnia magna* that varied slightly in time regarding the dilution that proved to immobilise most neonates after 48 h of exposure. *In situ* toxicity tests with the same species and test regime confirmed this picture: while caged daphnids employed upstream of the discharge had little failure regarding survival, downstream of the effluent discharge all animals died within the 48 h of exposure. In a separate fractionation step (see above) the authors were able to attribute most of the observed toxicity to chlorine as the principle toxicant in the effluent.

Diamond and Daley (2000) were able to differentiate the picture on the predictive capability of whole effluent testing (WET) by reviewing data available for acute and chronic fish and daphnia whole effluent test data for the USA and relating these to assessments based on benthic macroinvertebrate inventories in various streams. The capacity of lab WET testing for predicting macroinvertebrate assemblages increased with frequency of WET tests being performed, with contribution of the discharger to the receiving water in terms of volume ratios, and when several types of tests were included in the assessments.

5.2 Single species sensitivity distributions (SSDs)

Instead of focussing on selected single numbers of single species to derive a prediction or assessment on the hazard imposed by a contamination, approaches have been developed that use more of the available information in probabilistic ways, i.e. basically regarding exposure and effect information as probability distributions. The biological reasoning for this derives from the view that the biological components of ecosystems might be regarded as assemblages of different species. Furthermore, it is widely assumed that there is no single most sensitive species regarding responsiveness to toxicants. Instead regarding responses of different species to toxicant exposure by modelling distribution of species sensitivities to a given chemical has been brought forward by Kooyman (1987) and van Straalen and Denneman (1989). Subsequently, several teams considered specific aspects of the occurrence of biological variances in response to toxicant exposure (Behra et al., 1999,

Boutin and Rogers, 2000, Okkerman et al., 1991, McDaniel and Snell, 1999, Wagner and Lokke, 1991).

Two examples of the methodology are shown for the toxicity of the metal Cd using NOEC data for various soil organisms and for the toxicity of the insecticide lindane using NOEC values for aquatic organisms (figure 13, Traas et al., 2002). It can be seen that the unspecific toxicity of the metal results in a continuous distribution along the log concentration scale, which may be easily modelled by e.g. a logistic distribution function. Lindane on the other hand as a specifically acting insecticide produces jumps in the distribution, as non-target organisms will show clearly higher effect concentrations compared to arthropods. Depending on the goal one may even model this situation by employing different distribution functions for different taxa. Even clearer this picture may be derived for herbicides such as atrazine (de Zwart, 2002). Two characteristic values suitable for risk assessment or prediction are also shown in figure 14. When there is agreement on an intended level of protection in this example 95% of the species are to be protected (HC5 – hazardous concentration for 5% of the species in panel A) it is straightforward to estimate the corresponding concentration from the functional description. Vice versa, if an environmental concentration of a contaminant in the environment is known a potentially affected fraction of species (PAF in panel B) may be derived. The various uses of the species sensitivity distributions in ecotoxicological risk assessment have just been compiled in a monograph by Posthuma, Suter and Traas (2002).

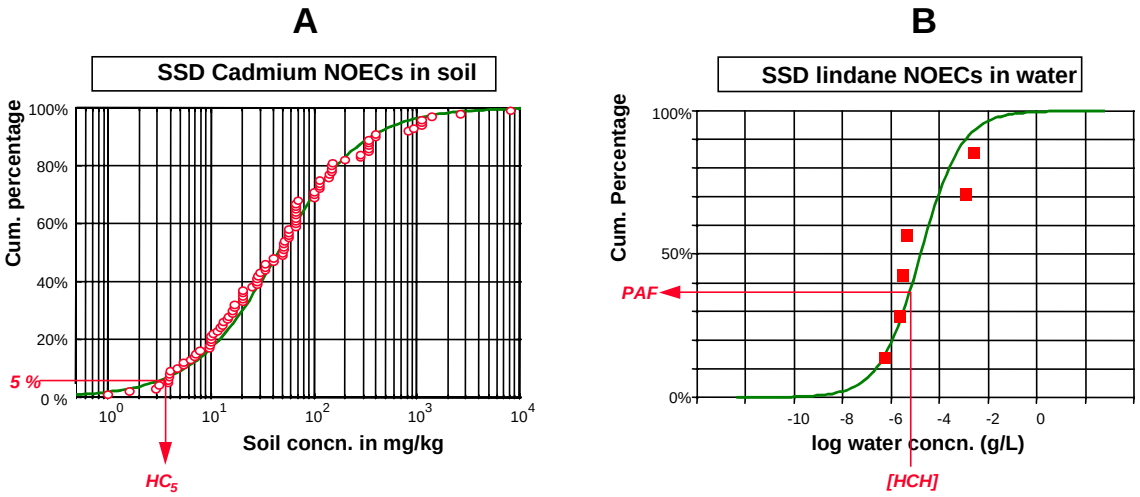


Figure 14: The use of species sensitivity distributions (SSDs) for deriving risk limits such as a certain hazardous concentration (HC_5 in panel A) and for risk assessment using potentially affected fractions of species (PAF in Panel B) (with kind permission from Traas et al., 2002).

While there is widespread acceptance of the achievements of this type of probabilistic approach to chemical hazard assessment, one has to be aware of different exposure profiles on a landscape scale due to different feeding strategies, discussed for example for bumblebees as compared to the standard test organism honeybee (Thompson and Hunt, 1999). This aspect by not is to be modelled by species sensitivity distributions but matters e.g. in pesticide non-target assessments. A major technical drawback of the SSD concept is

the requirement for sufficient sets of available experimental data for different species for a given toxin. Also, there are issues raised reflecting the fact that species sensitivity distributions are modelled on the basis of individual level effect parameters which only for populations with growth rates of about one will more or less mirror effects on populations (Forbes et al., 2001). Thus, including information on population dynamics could be an issue of further development in order to avoid undue over- or underestimations of risks.

5.3 Comparison of responses from single and multispecies testing

Several studies were performed to compare the effects of toxicants in single species tests and mircro- or mesocosms directly, of which a few will be cited here in their main conclusions.

Traunspurger et al. (1996) evaluated the effects of the herbicid isoproturone in single species tests and mesocosms. They noticed a higher sensitivity of the laboratory tests and concluded, that theses tests are sensitive instruments for screening possible effects, though no effect concentrations in the mesocosms were measured in this study. Jak et al. (1996) added a strain of *Daphnia magna* from the laboratory to a lake mesocosm study, to evaluate the effects of metals. This strain showed the same EC_{50} in the mesocosms as evaluated in a single species test. On the other hand he noticed, that species from the ecosystem were more sensitive and concluded that accurate safety factors must be considered to avoid the replacement of sensitive species and shifts in ecosystem function and structure. Rand et al. (2000) used three approaches for a risk assessment of the compound pyridaben. They estimated the environmental concentration (EEC) of the chemical and performed acute and chronic single species tests. By combining this data in a species sensitivity distribution, they evaluated, that there was a high risk for the most sensitive species from laboratory tests (based on EC_5 level). On the other hand their outdoor studies showed a weaker sensitivity than the labotaroty studies, resulting in a water-effect ratio of 18-24. They concluded that abiotic factors (photodegradation) reduced the bioavailability in the mesocosm study. Lampert et al. (1991) tested the effects of atrazine in systems of different complexity. Next to single species tests, artificial food chains and enclosure experiments were established. They showed, that the natural communities were the most sensitive and concluded, that the sensitivity of the systems increased with increasing complexity and that non-target-organisms (here daphnids) could be even more affected than the target organisms of the toxicant.

One important factor, changing the sensitivity of laboratory species tests and field populations is the development of tolerance. Ivorra et al. (2000) demonstrated, that a strain of the benthic diatom *G. parvulum* isolated from a stream, chronically subjected to high Zn (and Cd) contamination was more tolerant to Zn in the laboratory than the strain form an unpolluted stream. This tolerance was persistent for 2 years, suggesting a genetic based difference in tolerance. They concluded, that next to genetic adaptation tolerance might be related to different uptake rates or different intracellular pools of phytochelatins or

glutathione. Barata et al. (2000) compared the life history responses of field and laboratory populations of *Daphnia magna*, exposed to Cd and ethyl parathion. The results showed that the field population have a similar or greater tolerance to cadmium and ethyl parathion than the laboratory populations but the breadth of tolerance distribution was higher. The authors concluded, that tolerance is strongly influenced by genetic factors; the use of genetically homogeneous laboratory populations has limited relevance in predicting long-term responses of field populations to toxic chemicals, however, short-term responses seem better predictable.

A comprehensive review of critically evaluated literature on model ecosystems studies for assessing substances deleterious effects on biocoenosis and a comparison with data from a single species database (Länge et al., 1998) has been provided by ECETOC (ECOTOC, 1997). Data for 34 chemicals were evaluated using marine, static freshwater and flowing freshwater ecosystem models, with a bias towards the latter. These data were extracted out of 1108 original literature references selected for provision of NOEC and LOEC data. The main findings are aggregated in figure 15. The median ratio for NOEC values derived from single species studies to model ecosystem investigations was 1.5 and 8.1 for the 90%centile for all 34 chemicals using always most sensitive species or endpoints respectively. This clearly demonstrates that regulatory procedures using data for most sensitive species plus an additional assessment factor can be regarded as rational regarding the available evidence.

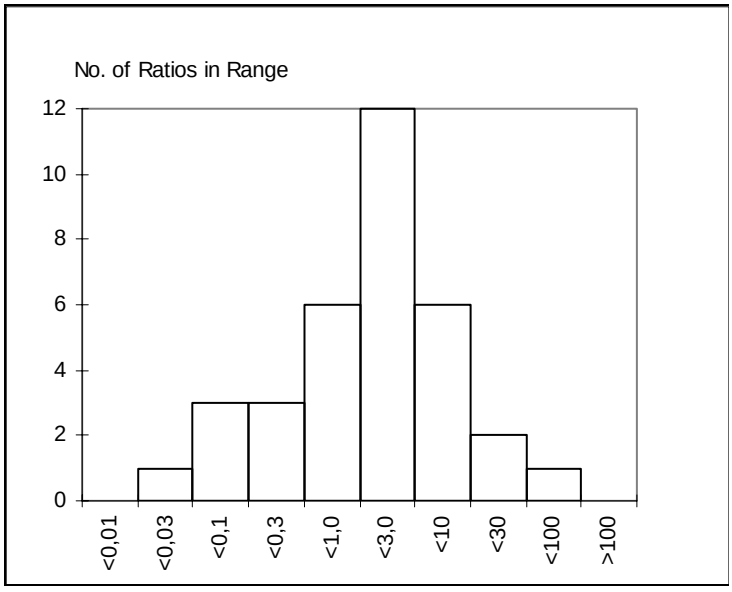


Figure 15: Ratios of single species NOECs to model ecosystem NOECs for different chemical classes (from ECETOC, 1997).

5.4 Ecological considerations

Using effect data generated for specific chemicals using single species for assessing or predicting effects for ecosystems commonly relies on comparison of predicted exposure concentrations for a specific media and lowest estimated no effect concentrations for a specified set of biotests. Many regulatory schemes use this procedure of a hazard quotient approach directly in form of so-called PEC/PNEC (predicted environmental concentration/predicted no effect concentration) or TER (toxicity/exposure) ratios to use derived values in decision rules of tier one risk assessment. Ecological thinking regarding this assessment strategy now reflects the plausibility of the assumed exposure and effect concentrations taken as basis for assessment. Mainly dissipation processes in reducing exposure, ecotoxic relevance of specific effects, and the importance of recolonisation of habitats are discussed as issue modifying assessments based on single species considerations in higher tier risk assessment schemes (Campbell et al., 1999, CLASSIC, 2001).

Huber provides an early example reviewing and assessing the ecotoxicological relevance of atrazine in aquatic systems (Huber, 1993). Concerning the exposure estimations for atrazine he puts emphasis on the possibility of organisms to metabolise atrazine along different pathways, and highlights the biased sampling strategies underlying occurrence reports of atrazine in aquatic media. On the effect assessment side he raises the issue of ecosystem recovery, which he judges to occur very fast after exposure, so that his assessment of a relevant damage to aquatic ecosystems ends about one order of magnitude higher than provided on the basis of single species results (Huber, 1993). In view of its many years of application as a herbicide the author comes to conclude that for atrazine "the residual risk appear to be relatively low and easy to predict" (Huber, 1993).

Suter et al. (1999) undertook a site specific ecological assessment concerning the risk for a given fish population in the Clinch River, exposed to a variety of metals and PCB contaminations mainly due to activities of the U.S. Department of Energy on its Oak Ridge Reservation in Tennessee. Biological survey data for different sites revealed in comparison to an ecological similar and relatively uncontaminated creek that all sites showed lower fish species richness and abundance. Multiple lines of evidence where then followed to investigate whether these community impairments could be detected and assessed unambiguously by other methods. Histopathological and reproductive bioindicators, ambient water toxicity estimated in development tests with fish eggs of medaka and redbreast channel catfish as well as short-term toxicity tests with *Ceriodaphnia* and fathead minnow, lethal body burdens measurements, and analysis of selected metals in the aquatic medium were used for that purpose. None of these lines used separately provided unambiguous explanation for a significant risk, though all were indicative for at least periodic events of toxic contamination at one particular site, the Poplar creek embayment. Weighing the evidence subsequently in what could be called an eco-epidemiological approach, made plausible that the community survey results were consistent with a significant toxic effect at the Poplar creek embayment site though habitat influence could not be completely discarded. Similar

conclusions about the necessity to connect independent lines of evidence, i.e. combine chemical and toxicological information to identify sites with ecotoxic levels of contamination seen at population level, were drawn from the investigations of the benthic invertebrates in the same area (Jones et al., 1999).

A different line of ecological reasoning looks at indirect effects of contaminants on communities in ecosystems. If such effects occur in the sense that the effect is truly dependent on the interaction between species e.g. in a food web, there is no point in attempting to predict this by single species considerations. Other effects are, however related to differential sensitivities against toxicants in communities. These in turn may affect community composition by selecting for more tolerant species. Altered composition structures and functions may be detected by suitable methodologies such as for example the pollution induced community tolerance (PICT) (Blanck and Dahl, 1996, Blanck et al., 1988, Rutgers and Breure, 1999). In an early example Blanck and Dahl were able to demonstrate that shifts in marine periphyton community tolerance against exposure to TBT from ship antifouling paints reflected altered community compositions. Moreover, these effects were detectable at concentrations below those which could be predicted by surrogate species testing (Blanck and Dahl, 1996). The notion that changes in community structure are to be assessed as deleterious has been challenged on the basis that functional replacement of one species by another in a community might be regarded as ecologically acceptable (Heger et al., 2001). This perception of functional redundancy as a recovery potential is however disputed (Rutgers and Breure, 1999, Blanck et al. 1988).

The principal limitations of any ecologically oriented assessment are manifold: Ecosystems are usually unique, i.e. assessments made for one contaminant in a specific system cannot easily be inferred to other systems. Ecological considerations like functional replacement or recolonisation potencies are not consented criteria in chemical risk assessment unlike lethality or reproductive disturbances for individuals or populations. To develop these will be a long process as can be learned for the debate on adverse effects of relevance in human toxicology. Finally, any statements on acceptability or negligibility of residual risks are not scientifically based. It needs simple thought to see that the residual risk is what we do not consider and therefore we cannot make statements as to its quantity or predictability. Of course, one may speculate or provide political judgements, but this should be clearly stated.

Besides widespread empirical evidence, there are theoretical considerations on the possibility of effect predictions for ecosystems. One important application of single species tests, used to assess and predict effects of chemicals on ecosystems are their implementation into models of the population dynamics in ecosystems. Model parameters are e.g. the growth rate, birth rate, respiration rate or death rate of a species, exposed to a chemical or the grazing pressure of a predator. These parameters, derived from laboratory tests for several chemicals could be inserted in a model, simulating species interaction or simple ecosystem features, e.g. seasonal temperature effects. Such models could then calculate direct and synergistic effects in the ecosystem, deriving from species interaction of different trophic levels.

When looking for effects at larger scales in time or space, experimental approaches are often not practicable. Especially in complex ecosystems or when diffuse contamination at low doses but longer time periods have to be considered, disturbances often could first be detected after long time periods. Scales of investigation should then be considered in the context of generation times of the species under observation or of vegetation periods or abiotic cycles of the ecosystem. These time scales, often comprising several years, cannot be recorded with a mesocosm approach. Corresponding field studies are even more time consuming and often have a retrospective character. Modelling effects of chemicals on ecosystems offer one practicable and often the only opportunity, to make prognoses in longer time scales.

In ecosystems, exposed to greater seasonal oscillations in contamination, the timing of a contamination could be of importance, when predicting xenobiotic effects. Such time courses could be e.g. an annual temperature effect or migration events of a population. In simulation approaches, the sensitive periods of the ecosystem could be detected, improving the efficiency of monitoring programs.

On the other hand, the complexity of most ecosystems requires reductionistic approaches, not considering all parameters of relevance in one simulation. So results of simulations must be taken with caution for deterministic predictions. They can be used as a prognostic tool that reveals possible reactions of the ecosystem and offer the opportunity, to expose effects, which have not been considered, yet. It is their advantage to work out, which abiotic parameters or which level of organisms will be important for predictions. They could give hints, what must be investigated and in which scales of time and space must be observed, so being a useful tool, when planning new experimental designs.

Further models, based on one set of data, cannot implicate all possible accidents. Computer based multiple simulations or the use of stochastic models are necessary to calculate the probability of effects, that could be expected. One possible tool, to consider the uncertainty of the model parameters, are Monte-Carlo-simulations. They do not base on fixed model parameters but on the probability of these values.

As an example Seitz and Ratte (1991) and Seitz and Poethke, (1995) developed a simulation model for pelagic systems of a deep dimictic lake. The aim of the study was, to derive a prognostic model, which reveals the potential reactions of this ecosystem to xenobiotics on the level of organisms. They reduced their system to two groups of algae and zooplankton and one fish group. For abiotic parameters, seasonal variations of temperature, light and the nutrients phosphate and nitrate were implicated. As input data to the model stress reactions of the organisms, caused by toxicants (e.g. increased respiration rates of cladoracern; decreased photosynthetic rates of algae) were considered. Their investigations showed several generalisable results, in which case models of population dynamics in ecosystems could help to understand effects of xenobiotics. The expectations of Seitz et al. (1995) were confirmed in that herbicides reduced the primary production of the phytoplankton and insecticides increased the respiration rates of zooplankton and, as an indirect effect the

biomass of algae. These results were clear, when the simulation period was one year (one vegetation period). However, simulating over 10 years revealed contradicting results, where the population dynamics of the observed species developed against this hypothesis, approaching a chaotic system. This demonstrated, that conventional test systems e.g. micro- or mesocosms are limited, to find such results.

5.5 What is the question?

Summarising, what has been laid out above, one may conclude from the review of current literature that all evidence so far shows, that single species data on the toxicity of pollutants can be used to predict the potential of adverse effects in ecosystems. There is no evidence that complex model ecosystems are systematically more or less sensitive to toxicants than single species tests. Higher variation in ecosystem as opposed to single species studies and the question of appropriate observation parameters for comparing different systems may cause technical difficulties in determining low effect concentrations. Additional problems arise in determining the correct concentration scale, as the environmental milieu effective in ecosystems may greatly alter the bioavailability of toxicants and may therefore show apparent lower toxicity. Also, as single species investigation commonly employ physiologically optimised conditions, effects of environmental factors on the sensitivity of species responses are easily overlooked. Principal limitations for extrapolation emerge when longer time scales are of concern or when structures or processes above the level of populations are affected. Additionally, ecological issues considered from a recovery perspective like recolonisation or functional replacement of species might modify assessment views.

The major challenge for an appropriate use of the many techniques available to gain single species information and the derivation of consistent assessments for ecosystems is to develop the right question for a predictive effort. This concern is illustrated in table 3 which tries to distinguish commonly found goals for prediction in studies on contaminants effect and allocates priorities to various criteria regarding the suitability of a given biomonitor for an anticipated purpose.

Three thoughts for future perspectives to improve and refine the use of single species investigations for the assessment and prediction of adverse effects of chemicals in ecosystems shall conclude this chapter. (i) Laboratory studies using single species need to be more precise in what they want to predict in term of ecosystem structure and function which hopefully leads to more focussed instrumentation and approaches. (ii) Prediction and assessment of the effects of contaminants on ecosystems should always be regarded as a process. There is no end in itself, thus new evidence or theoretical considerations should be incorporated for adjustment or improvement. Practically speaking, when there is a requirement to assess a specific chemical at a given time, say in a pesticide admission process, we should not trust our prediction efforts in risk assessment more than a weather forecast, but instead acknowledge our ignorance accordingly in risk management and install

appropriate monitoring tools to correct for false positive evidences coming up. (iii) Intelligent experimentation should replace too much standardised protocol testing.

Table 3: Matrix of application goals and suitability criteria for the utilisation of single species biotest systems.

	Suitability criteria for selection of a bioindicator, biomonitor, or biotest							
Goals for prediction or assessment	fast and easy	high repro-ducibility	accuracy	precision	high detection specificity	high detection sensitivity	scope for inference	validation of effect quality
Remediation need	o	-	+	-	+	o	-	o
Remediation success	-	+	+	0	0	+	+	-
Identification of causes of ecotoxicity	o	o	+	-	+	-	o	+
Comparison of chemicals	+	+	o	+	-	-	o	-
Mode of toxic action	-	-	+	-	+	o	+	+
Identification of vulnerable ecosystem structure or function	-	-	+	-	o	o	+	+
Effects of low contaminant concentration / chronic impact assessment	o	o	+	o	-	+	o	o
Complex sample contamination / combined effects	o	o	o	+	o	o	o	+
Routine surveillance	+	o	-	+	o	+	o	-

+ important, o valuable, - less important

Acknowledgements

The constructive criticism of the manuscript provided by Mechthild Schmitt-Jansen, Matthias Liess, and L Horst Grimme is kindly acknowledged.

Many colleagues have contributed in shared projects and hot discussions among others Thomas Backhaus, Hans Blanck, Wolfgang Bödeker, Werner Brack, Uwe Ensenbach, Beate

Escher, Gudrun Fahl, Michael Faust, L. Horst Grimme, Matthias Grote, Monika Knops, Andreas Kortenkamp, Eberhard Küster, Raik Meene, Ernst A. Nusch, Gerald Pöch, Peter Popp, Leo Posthuma, Beata Praszczyk, Toni Ratte, Heike Schmidt, Mechthild Schmitt-Jansen, Martin Scholze, Gerrit Schüürmann, Helmut Segner, Holger Weiß. Also, over the years there had been tremendous support in experimentation by Silke Aulhorn, Falk Dorusch, Margit Petre, Uta Kayser, Janet Krüger, and Svea Reiners. Sorry, whoever I forgot.

This contribution draws from experience of several projects of which explicitly the following are gratefully acknowledged: Ökotoxikologische Testbatterie (DBU, 40-111422), PREDICT (*ENV4-CT96-0319;* http://www-user.uni-bremen.de/~predict/), BEAM (*EVK1-1999-00055;* http://www.aquatox.uni-bremen.de/beam/*)*, and SAFIRA (BMBF, O2WT9949/3, http://safira.pro.ufz.de/).

References

Altenburger, R., Backhaus, T., Boedeker, W., Faust, M., Scholze, M., Grimme, L.H. 2000. Predictability of the toxicity of multiple chemical mixtures to *Vibrio fischeri*: Mixtures composed of similarly acting chemicals. Environ Toxicol Chem, 19:2341-2347.

Altenburger R., Backhaus, T. 2000. Der Faktor Zeit bei der Beurteilung von biologischen Wirkungen. In: Mücke W., Link, W. (eds) Biotests in der Praxis, Institut für Toxikologie und Umwelthygiene, Technische Universität München. pp. 61-74.

Altenburger, R., Bödeker, W., Faust, M., Grimme, L.H. 1990. Evaluation of the isobologram method for the assessment of mixtures of chemicals. Combination effect studies with pesticides in algal biotests. Ecotoxicol Environ Saf, 20:98-114.

Altenburger, R., Bödeker, W., Faust, M., Grimme, L.H. 1993. Aquatic Toxicology, Analysis of combination effects. In: Corn M (Ed) Handbook of hazardous materials. Academic Press. pp. 15-27.

Altenburger R, Bödeker W, Faust M, Grimme L H: 1996. Regulations for combined effects of pollutants: Consequences from risk assessment in aquatic toxicology. Food and Chemical Toxicology 34, 1155-1157.

Altenburger, R., Callies, R., Grimme, L.H., Mayer A., Leibfritz, D. 1995. The mode of action of glufosinate in algae: The role of uptake and ammonia assimilation pathways. Pesticide Science, 45, 305-310.

Altenburger, R., Nendza, M., Schüürmann, G. submitted. Mixture toxicty and its modeling by quantitative structure-activity relationships. Environ Toxicol Chem.

Anon., 1992. Report of the United Nations Conference on the Human Environment, Stockholm, 5-16 June 1972 (United Nations publication, Sales No. E.73.II.A.14 and corrigendum).

Babu, T.S., Marder, J.B., Tripuranthakam, S., Dixon, D.G., Greenberg, B.M. 2001. Synergistic effects of a photooxidized polycyclic aromatic hydrocarbon and copper on photosynthesis and plant growth: Evidence that in vivo formation of reactive oxygen species is a mechanism of copper toxicity. Environ Toxicol Chem 20, 1351-1358.

Backhaus, T., Altenburger, R., Boedeker, W., Faust, M., Scholze, M., Grimme, LH. 2000. Predictability of the toxicity of a multiple mixture of dissimilarily acting chemicals *to Vibrio fischeri*. Environ Toxicol Chem, 19, 2348-2356.

Backhaus, T., Froehner, K., Altenburger, R., Grimme, L. H. 1997: Toxicity testing with *Vibrio fischeri*: A comparison between the long term (24 h) and short term (30 min) bioassay. Chemosphere, 35, 2925-2938.

Barata, C., Baird, D.J., Markich, S.J.1999. Comparing metal toxicity among *Daphnia magna* clones: An approach using concentration-time-response surfaces. Arch Environ Contam Toxicol, 37, 326-331.

Barata, C., Baird, D.J. 2000. Determining the ecotoxicological mode of action from measurements made on individuals: results from instar-based tests with *Daphnia magna* Straus. Aquatic Toxicol 48, 195-209.

Basak, S.C., Grunwald, G.D., Host, G.E., Niemi, G.J., Bradbury, S.P. 1998. A comparative study of molecular similarity, statistical, and neural methods for predicting toxic modes of action. Environ Toxicol Chem 17, 1056-64.

Behra, R., Genomi, G.P., Joseph, A.L 1999. Effect of atrazine on growth, photosynthesis, and between-strain variability in *Scenedesmus subspicatus* (Chlorophyceae). Arch Environ Contam Toxicol, 37, 36-41.

Berenbaum, M.C.. 1981. Criteria for analysing interactions between biologically active agents. Adv Cancer Res, 35, 269-335.

Berenbaum, M.C. 1985. The expected effect of a combination of agents: the general solution. J Theor Biol, 114, 413-431.

Berenbaum, M.C. 1989. What is synergy? Pharmacol Rev, 41, 93-141.

Betts, K. S. 1998. Chemical industry pressured to test high-production volume chemicals. Environ Sci Technol 32, 251A.

Blanck, H, Dahl, B., 1996. Pollution-induced community tolerance (PICT) in marine periphyton in a gradient of tri-*n*-butyltin (TBT) contamination. Aquatic Toxicol, 35, 59-77.

Blanck, H, Wängberg, S.-A., Molander, S., 1988. Pollution-induced community tolerance – a new ecotoxicological tool. In: Cairns, J.J., Pratt, J.R. (Eds.), Functional Testing of aquatic biota for estimating hazards of chemicals. ASTM STP 988. American Society for testing materials, Philadelphia, pp. 219-230.

Boening, D.W. 1998. Toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin to severl ecological receptor groups: A short review. Ecotoxicol Environ Safety 39, 155-163.

Bol, J., Verhaar, H.J.M., van Leeuwen, C.J., Hermens, J.L.M. 1993. Predictions of the aquatic toxicity of high-production-volume-chemicals. Part A. Introduction and methodology. The Hague, Ministry of Housing, Physical Planing and Environment, Report 1993/9A.

Boutin C., Rogers, C.A. 2000. Pattern of sensitivity of plant species to various herbicides – an analysis with two databases. Ecotoxicology, 9, 255-271.

Brack, W., Altenburger, R., Ensenbach, U., Möder, M., Segner, H., Schüürmann, G. 1999. Bioassay-directed identification of organic toxicants in river sediment in the industrial region of Bitterfeld (Germany) - A contribution to hazard assessment. Arch Environ Contam Toxicol 37, 164-174.

Caisukant, Y., Yu, Q., Connell, D.W. 1999. The internal critical level concept of nonspecific toxicity. Res Environ Contam Toxicol 162, 1-41.

Campbell, P.J., Arnold, D.J.S., Brock, T.C.M., Grandy, N.J., Heger, W., Heimbach, F., Maund, S.J., Streloke, M. (eds.) 1999. Guidance document on higher-tier aquatic risk assessment for pesticides (HARAP). SETAC.

Caquet, T., Lagadic, L., Sheffield, S.R. 2000. Mesocosms in ecotoxicology (1): Outdoor aquatic systems. Rev Environ Contam Toxicol, 165, 1-38.

Chapman, P.M., Cadwel,l R.S., Chapman, P.F., 1996. A warning: NOECs are inappropriate for regulatory use. Environ Toxicol Chem 15:77-79.

Christensen, E.R. 1984. Dose-response functions in aquatic toxicity testing and the weibull model. Water Res. 18, 213-221.

De la Torre, F., Ferrari, L., Salibán, A. 2000. Long-term in situ toxicity bioassays of the Reconquista river (Argentina) water with *Cyprinus carpio* as sentinel organism. Water, Air and Soil Pollut, 121, 205-215.

Debus, R, Fliedner A, Schäfers C. 1996. An artificial mesocosm to simulate fate and effect of chemicals: Technical data and initial experience with the biocenosis. Chemosphere, 32, 1813-1822.

Desbrow, C., Routledge, E.J., Brighty, G.C., Sumpter, J.P, Waldock, M. 1998. Identification of estrogenic chemicals in STW effluents. 1. Chemical fractionation and in vitro biological screening. Environ Sci Technol, 32, 1549-1558.

de Zwart, D. 1995. Monitoring water quality in the future, Vol. 3 Biomonitoring. The Hague, VROM, 83.

de Zwart, D. in press. Observed regularities in species sensitivity distributions for aquatic species. in: Posthuma, L., Suter, G.W., Traas, T.P. (eds.) 2002. The use of species sensitivity distributions (SSD) in ecotoxicology. CRC Press, pp. 133-154.

Diamond, J., Daley, C. 2000. What is the relationship between effluent toxicity and instream biological condition? Environ Toxicol Chem, 19, 158-168.

Drent, J., Kersting, K.1993, Experimental ditches for research under natural conditions. Water Res, 27, 1497-1500.

ECETOC (European Centre for Ecotoxicology and Toxicology of Chemicals). 1997. The value of aquatic model ecosystem studies in ecotoxicology. Technical report no. 73. Brussels, ECETOC.

EEC (European Economic Community), 1990. Council Regulation on the evaluation and the control of the environmental risks of existing substances. COM (90) 227-final-syn 276.

EEC (European Economic Community), 1991. Council Directive 91/414/EEC of 15 July 1991 concerning the placing of plant protection products on the market. Official Journal L 230 , 19/08/1991 p. 0001 - 0032.

Escher, B., Hunziker, R.W., Schwarzenbach, R. 2001. Interaction of phenolic uncouplers in binary mixtures: Concentration additive and synergistic effects. Environ Sci Technol in press.

Escher, B., Hunziker, R., Schwarzenbach, R. 1999. Kinetic model to describe the intrinsic uncoupling activity of substituted phenols in energy transducing membranes. Environ Sci Technol 33, 560-570.

Escher, B., Hermens, J. submitted. Mechanisms in ecotoxicology: a crucial aspect in predicting the activity via QSAR and in the analysis of body burdens, species selectivity and mixture effects. Environ Sci Technol.

Fahl, G.M., Kreft, L., Altenburger, R., Faust, M., Bödeker, W., Grimme, L.H. 1995. pH-dependent sorption, bioaccumulation and algal toxicity of sulfonylurea herbicides. Aquatic Toxicology, 31, 175-187.

Faust, M., Altenburger, R., Backhaus, T., Blanck, H., Bodeker, W., Grammatica, P., Hamer, V., Scholze, M., Vighi, M., Grimme, L.H. 2001. Predicting the joint algal toxicity of multi-component s-triazine mixtures at low-effect concentrations of individual toxicants. Aquatic Toxicol. 56:13-32.

Faust, M., Altenburger, R., Backhaus, T., Blanck, H., Bodeker, W., Grammatica, P., Hamer, V., Scholze, M., Vighi, M., Grimme, L.H. submitted. Joint toxicity of 16 dissimilarily acting chemicals to the freshwater alga *Scenedesmus vacuolatus* is predictable by the concept of independent action. Aquatic. Toxicol.

Faust, M., Altenburger, R., Backhaus, T., Boedeker, W., Scholze, M., Grimme, L.H. 2000. Predictive Assessment of the Aquatic Toxicity of Multiple Chemical Mixtures. J. Eur. Qual, 29, 1063-1068.

Faust M, Altenburger R, Bödeker W, Grimme L H: 1994. Algal Toxicity of Binary Combinations of Pesticides. Bull. Environ. Contam. Toxcicol, 53, 134-141.

Forbes, V.E., Calow, P., Sibly, R.M. 2001. Are current species extrapolation models a good basis for ecological risk assessment? Environ Toxicol Chem, 20, 442-447.

Forbes, V.E., Forbes, T.L., 1994. Ecotoxicology in Theory and Practise. Chapman & Hall, London.

Ford, L. 2001. Development of chronic aquatic water quality criteria and standards for silver. Water Environ Res, 73, 248-253.

Girling, A.E., Tattersfield, L.J., Mitchell, G.C., Pearson, N., Woodbridge, A.P., Bennett, D. 2000. Development of methods to assess the effects of xenobiotics in outdoor artificial streams. Ecotoxicol Environ Safety, 45, 1-26.

Goser, B. 1997. Dichteabhängige Änderung der Entwicklung und reproduktion bei Cladoceren. Ursachen und ökologische Bedeutung. Shaker Verlag, Aachen. pp. 210.

Greco, W., Bravo, G., Parsons, J.C. 1995. The search for synergy: A critical review from a response surface perspective. Pharmacol Rev, 47, 331-385.

Grimme L H, Faust M, Bödeker W, Altenburger R. 1996. Aquatic toxicity of chemical substances in combination: Still a matter of controversy. Human Ecol Risk Assess, 2, 426-433.

Grimme, L.H., Rieß, M.H., Manthey, M., Faust, M., Altenburger, R: 1993. Cell Physiological Parameters to Detect Ecotoxicological Risks. Sci Total Environ, Suppl. 741-748.

Halpern, S., 1993. United Nations Conference on Environment and Development: Process and documentation. Academic Council for the United Nations System (ACUNS) reports and papers, no. 2. Providence, RI: Academic Council for the United Nations System (ACUNS).

Heger W, Brock TCM, Giddings J, Heimbach F, Maund SJ, Norman S, Ratte H-T, Schäfers C, Streloke M. 2001. Proceedings of the CLASSIC Workshop (Community Level Aquatic System Studies Interpretation Criteria). SETAC US Publication, in press.

Hermens, J., Broekhuyzen, E., Canton, H., Wegman, R. 1985 a. Quantitative structure-activity relationships and mixture toxicity studies of alcohols and chlorohydrocarbons: effects on growth of Daphnia magna. Aquat Toxicol 6:209-217.

Hermens, J., Busser, F., Leeuwangh, P., Musch, A. 1985 b. Quantitative structure-activity relationships and mixture toxicity studies of organic chemicals in Photobacterium *phosphoreum*: the Microtox test. Ecotoxicol Environ Saf, 9, 17-25.

Hermens, J., Canton, H., Janssen, P., de Jong, R. 1984 a. Quantitative structure-activity relationships and toxicity studies of mixtures of chemicals with anaesthetic potency: acute lethal and sublethal toxicity to Daphnia magna. Aquat Toxicol, 5, 143-154.

Hermens, J., Canton, H., Steyger, N., Wegman, R. 1984 b. Joint effects of a mixture of 14 chemicals on mortality and inhibition of reproduction of Daphnia magna. Aquat Toxicol, 5, 315-322.

Hermens, J., Leeuwangh, P., Musch, A. 1985 c. Joint toxicity of mixtures of groups of organic aquatic pollutants to the guppy (Poecilia reticulata). Ecotoxicol Environ Saf, 9, 321-326.

Hermens, J., Leeuwangh, P. 1982. Joint toxicity of mixtures of 8 and 24 chemicals to the guppy (Poecilia reticulata). Ecotoxicol Environ Saf, 6, 302-310.

Holten Lützhoft, H.C., Halling-Sorensen, B., Jorgensen, S.E. 1999. Algal Toxicity of Antibacterial Agents Applied in Danish Fish Farming. Arch. Environ. Contam. Toxicol, 36, 1-6.

Huber, W. 1993. Ecotoxicological relevance of atrazine in aquatic systems. Environ Toxicol Chem, 12, 1865-1881.

Ireland, D.S., Burton, G.A., Heiss, G.G. 1996. In situ toxicity evaluations of turbidity and photoinduction of polycyclic aromatic hydrocarbons. Environ Toxicol Chem, 15, 574-581.

Ivorra, N., Barranuet, C., Jonker, M., Kraak, M.H.S., Admiraal, W. (2000). Differences in Zn tolerance in strains of the freshwater microbenthic diatom *Gomphonema pavulum*. (submitted).

Jak, R.G., Maas, J.L., Scholten, M. C. Th. 1996. Evaluation of laboratory derived toxic effect concentrations of a mixture of metals by testing fresh water plantkon communities in enclosures. Water Res, 30, 1215-1227.

Jobling, S., Reynolds, T., White, R., Parker, M.G., Sumpter, J.P. 1995. A variety of environmental persistent chemicals, including some phthalate plarsticizers, are weakly estrogenic. Environ. Health Perspec. 103, 582-587.

Johnston, G., Walker, C.H., Dawson, A. 1994. Interactive effects of prochloraz and malathion in pigeon, starling and hybrid red-legged partridge. Environ Toxicol Chem, 13, 115-120.

Jones, D.S., Barnthouse, L.W., , Suter G.W., Efrroymson, R.A., Field, J.M., Beauchamp, J.J. 1999. Ecological assessment in a large river-reservoir: 3. benthic invertebrates. Eviron Toxicol Chem, 18, 599-609.

Kapur, S, Shusterman, A., Verma, R.P., Hansch, C., Selassie, C.D. 2000. Toxicology of benzyl alcohols: a QSAR analysis. Chemosphere, 41, 1643-1649.

Knops, M., Altenburger, R., Segner, H. 2001. Alterations of physiological energetics, growth and reproduction of *Daphnia magna* under toxicant stress. Aquatic Toxicol, 53, 79-90.

Kolkwitz, R, Marsson, M. 1902. Grundsätze für die biologische Beurteilung des Wassers nach seiner Flora und Fauna. Mitteilungen der königlichen Prüfanstalt Wasserversorgung Abwasserbeseitigung Berlin-Dahlem 1:33-72.

Kolkwitz, R. 1950. Ökologie der Saprobien. Über die Beziehungen der Wasserorganismen zur Umwelt. Schriftenreihe des Verbandes für Wasser-, Boden- und Lufthygiene 4:1-64.

Könemann H. 1980. Structure-Activity Relationships and Additivity in Fish Toxicities of Environmental Pollutants. Ecotoxicol Environ Saf, 4, 415-421.

Könemann H. 1981. Fish toxicity tests with mixtures of more than two chemicals: a proposal for a quantitative approach and experimental results. Toxicology, 19, 229-238.

Kooijman, S.A.L.M., Bedaux, J.J.M. 1996. The analysis of aquatic toxicity data. Amsterdam, VU University press, pp149.

Kooyman, S.A.L.M. 1987. A safety factor for LC50 values allowing for differences among species. Water Res, 21, 269-276.

Kortenkamp, A., Altenburger, R. 1998. Synergisms with mixtures of xenoestrogens - a reevaluation using the method of isoboles. Sci Total Environ, 221, 59-73.

Kortenkamp, A., Altenburger, R. 1999. Approaches to assessing combination effects of oestrogenic pollutants. Sci Total Environ, 233, 131-140.

Kreeb, K.H., (ed.), 1990. Methoden der Pflanzenökologie und Bioindikation. G. Fischer Verlag, Jena.

Länge, R., Hutchinson, T.H., Scholz, N., Solbé, J. 1998. Analysis of the ECOTOC aquatic toxicity (EAT) database II - Comparison of acute to chronic ratios for various aquatic organisms and chemical substances. Chemosphere, 36, 115-127.

Lampert, W., Fleckner, W., Pott, E., Schober, U., Störkel, K.U. 1991. Herbicide effects on planktonic systems of different complexity. Hydrobiologia, 188/189, 415-424.

Laskowski, R., 1995. Some good reasons to ban the use of NOEC, LOEC and related concepts in ecotoxicology. OIKOS, 73, 140-143.

Legierse, K.C.H.M., Verhaar, H.J.M., Vaes, W.H.J., de Bruin, J.H.M., Hermens, J.L.M. 1999. Analysis of time-dependent acute toxicity of organophosphorus pesticides: The critical target occupation model. Environ Sci Technol, 33, 917-925.

Levasseur, L.M., Slocum, H.K., Rustum, Y.M., Greco, W.R. 1998. Modelling of time-dependency of *in vitro* drug cytotoxicity and resistence. Cancer Res, 58, 5749-5761.

Lien, G.J., J.M. McKim, A.D. Hoffman, C. T. Jenson. 2001. A physiologically based toxicokinetic model for lake trout (*Salvelinus namaycush*). Aquatic Toxicology, 51.335-350.

Liess, M., Champeau, O, Riddle, M, Schulz, R, Duquesne, S. 2001. Combined effects of ultraviolett-B radiation and food shortage on the sensitivity of the Antarctic amphipod Paramoera walkeri to copper. Environ Toxicol Chem 20:2088-2092.

Liess, M., Schulz, R. 1999. Linking insecticide contaminationn and population response in an agricultural stream. Environ Toxicol Chem, 18, 1948-1955.

Lipnick, R.L. 1989. Narcosis, electrophile and proelectrophile toxicity mechanisms: Application of SAR and QSAR. Environ Toxicol Chem 8, 1-12.

Maltby, L., Clayton S.A., Yu, H., McLoughlin, N., Wood, R.M., Ying, D. 2000. Using single-species toxicity tests, community-level responses, and toxicity identification evaluations to investigate effluents impacts. Environ Toxicol Chem, 19, 151-157.

Marchini, S., Passerini, L., Hoglund, M.D., Pino, A., Nendza, M. 1999. Toxicity of aryl- and benzhalides to *Daphnia magna* and classification of their mode of action based on quantitative structure-activity relationship. Environ Toxicol Chem, 18, 2759-2766.

Matthiessen, P. 2000. Is endocrine disruption a significant ecological issue? Ecotoxicology, 9, 21-24.

McDaniel, M., Snell, T.W. 1999. Probability distributions of toxicant sensitivity for freshwater rotifer species. Environ Toxicol, 14, 361-366.

McKim, J.M., Bradbury, S.P., Niemi, G.J. 1987. Fish acute toxicity syndromes and their use in the QSAR approach to hazard assessment. Environ Health Perspec, 71, 171-186.

Milles, D., 1989. Grenzen natürlicher Selbstreinigung - Zur Geschichte medizinischer Grenzwertkonzepte. In: Kortenkamo, K., Grahl, B., Grimme, L.H., (Eds.), Die Grenzenlosigkeit der Grenzwerte. C.F. Müller, Karlsruhe, pp. 197-219.

Milles, D., 1991. Von Schädlingen und Schädigungen - Zur Geschichte der Pestizidzulassung. In: Rehbinder, E., (Ed.), Bremer Kolloquium über Pflanzenschutz (Tagungsband). Werner, Düsseldorf, pp. 17-43.

Molander, S., Dahl, B., Blanck, H., Jonsson, J., Sjöström, M. 1992. Combined effects of tri-n-butyl tin (TBT) and diuron on marine periphyton communities detected as Pollution induced community tolerance. Arch Environ Contam Toxicol 22, 419-427.

Moore, D.R.J., Caux, P-Y. 1997. Estimating low toxic effects. Environ Toxicol Chem, 16, 794-801.

Morton, M.G., Mayer F.L., Dickson, K.L., Waller, W.T., Moore, J.C. 1997. Acute and chronic toxicity of azinphos-methyl to two estuarine species, Mysidopsis bahia and Cyprinodon variegatus. Arch Environ Contam Toxicol, 32, 436-441.

Nendza, M., Hermens, J. 1995, Properties of chemicals and estimation methodologies. in van Leeuwen C.J. and J.L.M. Hermens. Risk Assessment of Chemicals: An Introduction. 1995. Dordrecht: Kluwer; pp 239-292.

Nendza, M., Müller, M. 2000. Discriminating toxicant classes by mode of action: 2. Physico-chemical descriptors. Quant Struct-Act Relat 19, 581-598.

Niculescu, S.P., Kaiser, K.L.E., Schultz, T.W. 2000. Modeling the toxicity of chemicals to *Tetrahymena pyriformis* using molecular fragment descriptors and probabilistic neural networks. Arch Environ Contam Toxicol, 39, 289-298.

Nusch, E.A. 1992. Grundsätzliche Vorbemerkungen zur Planung, Durchführung und Auswertung biologischer und ökotoxikologischer Testverfahren. in: Steinhäuser, K.G., Hansen, P.-D. Biologische Testverfahren. Schr. Reihe Verein WaBoLu 89, Gustav Fischer Verlag, Stuttgart, 35-48.

OECD (Organisation for economic co-operation and development), 1995. Report of the OECD Workshop on Environmental Hazard/Risk Assessment. OECD Environment Monographs No. 105, Paris.

OECD (Organisation for economic co-operation and development), 1998. Report of the OECD workshop on statistical analysis of aquatic toxicity data. OECD Series on testing and assessment, No. 10, Paris.

Okkerman; P.C., van der Plassche, E.J., Sloof, W., van Leeuwen, C.J., Canton, J.H. 1991. Ecotoxicological effects assessment: A comparison of several extrapolation procedures. Ecotoxicol Environ Safety, 21, 182-193.

Parkerton, T.F., Konkel, W.J. 2000. Application of quantitative structure-activity relationships for assessing the aquatic toxicity of phthalate esters. Environ Ecotoxicol Safety, 45,61-78.

Pereira, A.M.M., da Maia Soares, A.M.V., Goncalves, F., Ribeiro, R. 1999. Test chambers and test procedures for in situ toxicity testing with zooplankton. Environ Toxicol Chem, 18, 1956-1964.

Pöch, G. 1993. Combined effects of drugs and toxic agents. Modern evaluation in theory and practice. Springer Verlag, Wien, New York, USA.

Posthuma, L., Suter, G.W., Traas, T.P. (eds.) 2002. The use of species sensitivity distributions (SSD) in ecotoxicology. CRC Press.

Purdom, C.E., Hardiman, P.A., Bye, V.J., Eno, E.N., Tyler, C.R., Sumpter, J.P. 1994. Chem Ecol 8, 275-285.

Pyle, G.G., Swanson, S.M., Lehmkuhl, D.M. 2001. Toxicity of uranium mine-receiving waters to caged fathead minnows, *Pimephales promelas*. Ecotoxicol Environ Safety 48, 202-214.

Rand, G., Clark, J.R. 2000. Hazard/risk assessment of pyridaben: I. Aquatic toxicity and environmental chemistry. Ecotoxicology 9, 157-168.

Rand, G., Clark, J.R. 2000. Hazard/risk assessment of pyridaben: II. Outdoor aquatic toxicity studies and the water-effect ratio. Ecotoxicology 9, 169-177.

Rand, G.M., Wells P.G., Mc Carty L.S.. 1995. Introduction to aquatic toxicology. in: Rand G.M. (Ed.). Fundamentals of aquatic toxicology. 2nd ed. Taylor & Francis. pp.3-67.

Roex, E.W.M., van Gestel, C.A.M., van Wezel, A.P, van Straalen, N.M. 2000. Ratios between acute aquatic toxicity and effects on population growth rates in relation to toxicant mode of action. Environ Toxicl Chem, 19, 685-693.

Roex, E.W.M., van Gestel,C.A.M.; van Wezel, A.P.; van Straalen, N.M.. 2000. Ratios between Acute Aquatic Toxicity and Effects on Population Growth Rates in Relation to Toxicant Mode of Action. Environ Toxicol Chem, 19, 685-693.

Routledge, E.J., Shean, D., Desbrow, C., Brighty, G.C., Waldock, M., Sumpter, J.P. 1998. Identification of estrogenic chemicals in STW effluents. 2. In vivo responses in trout and roach. Environ Sci Technol 32, 1559-1565.

Rutgers, M., Breure, A.M. 1999. Risk assessment, microbial communities, and pollution-induced community tolerance. Human Ecol Risk Assess. 5: 661-670.

Sabliunas, D. 1999. Semipermeable membrane devices in monitoring of organic pollutants in the aquatic environment. PhD thesis, Lund University.

Schmitt H, Altenburger R, Jastorff B, Schüürmann G. 2000. Quantitative structure –activity analysis of the algae toxicity of nitroaromatic compounds. *Chem Res Toxicol* 13:441-450.

Scholze, M., Boedeker, W., Faust, M., Backhaus, T., Altenburger, R., Grimme, L.H. 2001. A general best-fit method for concentration-response curves and the estimation of low-effect concentrations. Environ Toxicol Chem. 20, 448-457.

Schüürmann G 1998. Ecotoxic modes of action of chemical substances. In: Schüürmann G, Markert B (eds) Ecotoxicology. John Wiley and Spektrum Akademischer Verlag, New York, USA, pp. 665-749.

Schweigert N, Acero JL, von Gunten U, Canonica S, Zehnder AJB, Eggen RIL. 2000. DNA degradation by the mixture of copper and catechol is caused by DNA-copper-hydroperoxo complexes, probably DNA-Cu(I)OOH. Environ Mol Mutagenesis, 36, 5-12

Schweigert N, Hunziker RW, Escher BI, Eggen RIL. 2001. Acute toxicity of (chloro-)catechols and (chloro-)catechol-copper combinations in Escherichia coli corresponds to their membrane toxicity in vitro. Environ Toxicol Chem, 20, 239-247.

Segner et al., personal communication

Seitz, A., Poethke, H.J. 1995. Strukturanalyse und Modellierung von Zoopolankton-Fisch-Freilandsystemen zur Bewertung von Fremdstoffwirkungen in aquatischen Ökosystemen. in: Kirchner, M., Bauer, H. (eds.) Proceedings des Statuseminars zum Förderschwerpunkt ”Ökotoxikologie” des BMBF.

Seitz, A., Ratte, H.T. 1991. Aquatic ecotoxicology: on the problems of extrapolation from laboratory experiments with individuals and populations to community effects on the field. Comp Biochem Physiol, 100: 301-304.

Shukla, R., Wang Q., Fulk, F., Deng, C. Denton, D. 2000. Bioequivalence approach for whole effluent toxicity testing. Environ Toxicol Chem 19,. 169-174.

Soucek, D.J., Cherry, D.S., Currie, R.J., Latimer, H.A., Trent, G.C. 2000. Laboratory to field validation in an integrated assessment of an acid mine drainage-impacted watershed. Environ Toxicol Chem, 19, 1036-1043.

Steinhäuser, K.G., Hansen, P.-D. (eds). Biologische Testverfahren. Schr. Reihe Verein WaBoLu 89, Gustav Fischer Verlag, Stuttgart, pp. 879.

Suter G.W., Barnthouse, L.W., Efrroymson, R.A., Jager, H. 1999. Ecological assessment in a large river-reservoir: 2. fish community. Eviron Toxicol Chem, 18, 589-598.

Thomson, H.M., Hunt, L.V. 1999. Extrapolating from honeybees to bumblebees in pesticide risk assessment. Ecotoxicology 8, 147-166.

Tong, W., Lowis, D.R., Perkins, R., Chen, Y., Welsh, W.J., Godette, D.W., Heritage, T.W., Sheehan, D.M. 1998. Evaluation of quantitative strucutre-activity relationship methods for large-scale prediction of chemicals binding to the estrogen receptor. J Chem Information Comp Sci, 38, 669-677.

Trauspurger, W., Schäfer, H., Remde, A. 1996. Comparative investigation on the effect of a herbicide on aquatic organisms in single species tests and aquatic microcosms. Chemosphere, 33, 1129-1141.

Traas, T.P., van de Meent, D., Posthuma, L., Hamers, T.H..M., Kater, B.J., de Zwart, D., Aldenberg, T. 2002. The potentially affected fraction as a measure of ecological risk. in: Posthuma, L., Suter, G.W., Traas, T.P. (eds.) The use of species sensitivity distributions (SSD) in ecotoxicology. CRC Press pp. 315-344.

Tyler, C.R., Jobling, S., Sumpter, S.P. 1998. Endocrine disruption in wildlife: A critical review of the evidence. Critical Rev Toxicol, 28: 319-361.

Vaal, M., van der Wal, J.T., Hermens, J., Hoekstra, J. 1997a. Pattern analysis of the variation in the sensitivity of aquatic species to toxicants. Chemosphere 35, 1291-1309.

Vaal, M., van der Wal, J.T., Hoekstra, J., Hermens, J. 1997b. Variation in the sensitivity of aquatic species in relation to the classification of environmental pollutants. Chemosphere 35, 1311-1327.

Vaal, M.A., van Leeuwen, C.J., Hoekstra, J.A., Hermens, J.L.M. 2000. Variation in sensitivity of aquatic species to toxicants: Practical consequences for effect assessment of chemical substances. Environ Management, 25, 415-423.

van Leeuwen, C.J., van der Zandt, P.T.J., Aldenberg, T., Verhaar, H.J.M., Hermens, J.L.M. 1992. Application of QSARs, extrapolation and equilibrium partioning in aquatic effect assessment. I. Narcotic industrial pollutants. Environ Toxicol Chem, 11,267-282.

van Straalen, N.M., Denneman, C.A.J. 1989. Ecotoxicological evaluation of soil quality criteria. Ecotoxicol Environ Saf 18, 241-251.

Van Wijk, D.J., Hutchinson, T.H. 1995. The ecotoxicity of chlorate to aquatic organisms: A critical review. Ecotoxicol Environ Safety, 32, 244-253.

van Wijngaarden, R.P.A., Van der Brink, P.J., Crum, S.J.H., Voshaar, J.H.O., Brock, T.C.M., Leeuwangh, P. 1996. Effect of the insecticide Dursban® 4E (active ingriedient chlopyrifos) in outdoor experimental ditches:I. Comparison of short-term toxicity between the laboratory and the field. Environ Toxicol Chem, 15, 1133-1142.

Verhaar, H.J.M., van Leeuwen, C.J., Hermens, J. 1992. Classifying environmental pollutants. 1: Structure-activity relationships for prediction of aquatic toxicity. Chemosphere, 25, 471-491.

Vighi M, Altenburger R, Arrhenius Å., Backhaus T, Bödeker W, Blanck H, Consolaro F, Faust M, Finizio A, Froehner K, Gramatica P, Grimme LH, Grönvall F, Hamer V, Scholze M, Walter H. in press. Water quality objectives for mixtures of toxic chemicals: problems and perspectives. Ecotoxicol Environ Safety

Wagner, C., Lokke, H. 1991. Estimation of ecotoxicological protection levels from NOEC toxicity data. Water Res, 25, 1237-1242.

Walter H, Consolaro F, Gramatica P, Scholze M, Altenburger R. accepted. Mixture toxicity of priority pollutants at no observed effect concentrations (NOECs). Ecotoxicology

Wells, P.G., Lee, K., Blaise, C. (eds) 1998. Microscale testing in aquatic tocixology. CRC Press: Boca Raton pp 679.

Wenzel, A. Nendza, M., Hartmann, P., Kanne, R. 1997. Testbattery for the assessment of aquatic toxicity. Chemosphere 35, 307-322.

Yang, R., Thurston, V., Neuman, J.. 2000. A physiological model to predict xenobiotic concentration in fish. Aquatic Toxicology, 48, 109-117.

Xing, L., Welsh, W.J., Tong, W., Perkins, R., Sheehan, D.M. 1999. Comparison of estrogen receptor alpha and beta suntypes based on comparative molecular field analysis (CoMFA). SAR QSAR Environ Res, 10, 215-230.

Kapitel II

Bioassay-directed identification of organic toxicants in river sediment in the industrial region of Bitterfeld (Germany) – A contribution to hazard assessment.

Werner Brack, Rolf Altenburger, Uwe Ensenbach, Monika Möder, Helmut Segner und Gerrit Schüürmann
Archives of Environmental Contamination and Toxicology, 37, 164-174. (1999)

Arch. Environ. Contam. Toxicol. 37, 164–174 (1999)

ARCHIVES OF
Environmental
Contamination
and Toxicology

Bioassay-Directed Identification of Organic Toxicants in River Sediment in the Industrial Region of Bitterfeld (Germany)—A Contribution to Hazard Assessment

W. Brack,[1] R. Altenburger,[1] U. Ensenbach,[1] M. Möder,[2] H. Segner,[1] G. Schüürmann[1]

[1] UFZ Centre for Environmental Research Leipzig-Halle, Department of Chemical Ecotoxicology, Permoserstraße 15, 04318 Leipzig, Germany
[2] UFZ Centre for Environmental Research Leipzig-Halle, Department of Analytical Chemistry, Permoserstraße 15, 04318 Leipzig, Germany

Received: 11 November 1998/Accepted: 14 March 1999

Abstract. Bioassay-directed identification of toxicants in an acetonic extract of a sediment of the riverine Spittelwasser in the industrial region of Bitterfeld (Germany) was conducted. For this purpose, a combination of chromatographical fractionation, chemical analysis, and a biotest battery including *Vibrio fischeri* (inhibition of bioluminescence), *Daphnia magna* (immobilization), and *Scenedesmus vacuolatus* (inhibition of cell multiplication) was applied. Major toxicants identified and confirmed were methyl parathion (*D. magna*), prometryn, N-phenyl-β-naphthalene amine, PAHs (*S. vacuolatus*), and tributyltin (all biotests). Toxicity to *V. fischeri* was dominated by elemental sulfur. Results indicate high toxicant loads in the sediment about 7 years after closedown of a majority of chemical production sites at Bitterfeld. Comparison of potential exposure and toxicity data indicate a severe hazard potential to aquatic organisms due to organic toxicants. The results illustrate the potency of a biotest battery for identification of toxicants in contaminated sediment within the frame of toxicity identification procedures.

The highly industrialized region of Bitterfeld in Sachsen-Anhalt, Germany, is considered to be one of the most contaminated areas in Europe (Kuballa *et al.* 1995). For several decades, untreated effluents from chemical plants containing a broad range of organic and inorganic contaminants were discharged via the small riverine Spittelwasser to the River Mulde, a tributary to the River Elbe. For that reason, Spittelwasser and Mulde sediments are reported to be contaminated with a broad range of potentially toxic compounds such as hexachlorocyclohexane (HCH) isomeres, 2,2-bis(p-chlorophenyl)-1,1,1-trichloroethane (DDT) and degradation products, polyaromatic hydrocarbons (PAHs), chlorophenols, organotin compounds, and heavy metals (Popp *et al.* 1994; Neumeister and Ruske 1995; Kuballa *et al.* 1995).

Despite chemical analysis of sediments and soils flooded by the Rivers Spittelwasser and Mulde, to date there is no information available about potential toxic effects of this mixture of contaminants. Using chemical analysis alone, however, toxic potency of complex environmental samples is difficult to predict because important compounds may be overlooked, the relative ecotoxicological relevance of single contaminants in complex mixtures is difficult to predict, and for many compounds no sufficient toxicity data are available. Thus, direct measurement of potential effects on biota using biotests is necessary. However, biotests as a rule do not provide information about the compounds eliciting the measured effects, which is necessary for hazard assessment of contaminated environmental compartments. Therefore, a tool is necessary for providing toxicity data as well as an identification of the compounds causing the effects. In this context, the most powerful tools for the identification of toxicants in environmental samples are combinations of biotests, biotest-directed fractionation, and chemical analysis (Samoiloff *et al.* 1983; Schuetzle and Lewtas 1986; Grifoll *et al.* 1990; Fernández *et al.* 1992; Ho and Quinn 1993). A special procedure for toxicity identification evaluation (TIE) in aquatic samples was introduced by US EPA (Mount and Anderson-Carnahan 1989; Mount 1989; Norberg-King *et al.* 1991). Though aquatic samples can be directly subjected to TIE procedures, sediments and other solids need an extraction step providing a liquid sample, such as pore water, an aqueous elutriate, or an organic extract that can be fractionated.

The choice of the extraction methods for sediments depends on the objective of the study and the underlying concept of exposure of aquatic organisms to sediment-borne toxicants. This exposure may occur via pore water, ingested sediment, and direct contact with sediment particles. These pathways are not only important for benthic organisms (Harkey *et al.* 1994) but also for algae, *daphnids*, or fish in case of resuspension of the particles (Knezovich *et al.* 1987). Biotesting of pore water or aqueous elutriates neglects two of three possible pathways of exposure and may underestimate toxicity. A recent investigation about the bioavailability of fluoranthene in sediments to benthic organisms predicts a dietary uptake flux that is 20 to 30 times higher than that due to pore water only (Forbes *et al.* 1998). The investigation of organic extracts leaves out the question of bioavailability and tries to render a major part of organic sediment-bound contaminants available to biotesting and toxicity identification. Effect data from this approach are useful for the identification of potential organic toxicants in the sediment, but are no measure for the toxicity of the bulk sediment. For an

Correspondence to: W. Brack

estimation of the ecotoxicological relevance of the identified compounds in the respective sediment, additional data concerning their bioavailability are considered. Toxicity caused by inorganic or volatile compounds such as heavy metals, hydrogen sulfide, and ammonia is not considered in this approach.

The objectives of this study were to characterise the toxic potency of organic extracts of Spittelwasser sediment and to identify toxic compounds present in these extracts applying a combination of biotests with chromatographic fractionation and chemical analysis. It is evident that measured toxicities of sediments vary with the biotest applied (Athey *et al.* 1989). Therefore, a biotest battery was applied including luminescent bacteria, daphnia and green algae representing different trophic levels as well as different effect types such as acute inhibition of bacterial energy metabolism, acute invertebrate vitality and chronic toxicity on proliferation of photoautotrophic organisms. The ecotoxicological relevance of the identified toxicants is discussed using literature data concerning effects, physicochemical properties, sorption behaviour and degradability.

Materials and Methods

The applied procedure is based on four consecutive steps: (1) Extraction and detection of biological effects in the sediment extract; (2) chromatographic fractionation and detection of biological effects in the fractions; (3) identification and quantification of components of toxic fractions using gas chromatography with mass selective detection (GC/MSD); and (4) confirmation of toxicants applying biotests to identified compounds or mixtures of them.

Sampling and Sample Extraction

The sediment was sampled at a water depth of about 30 cm in a creek with slowly moving water in the riverine Spittelwasser in the plain of River Mulde. The sampling site is located north of the village of Jessnitz (Figure 1). With a sediment corer 12 cores of about 20 cm depth were drawn, mixed, and homogenized. The anaerobic sediment sample was air-dried and sieved through a mesh of 0.2 mm to remove stones and coarse and middle sand. After sieving, ignition loss and grain size composition was measured on pretreatment with sodium pyrophosphate according to DIN 19683 (1973) (Table 1).

The dried and sieved sample (400 g) was Soxhlet-extracted for 24 h with acetone. Extraction with acetone has been shown to give good recoveries for compounds of medium polarity like organophosphorous pesticides (Abd-Allah 1995) as well as for highly lipophilic compounds such as polycyclic aromatic hydrocarbons (Sun *et al.* 1998).

The extract volume was reduced to 500 ml in a rotary evaporator. An aliquot of 200 ml was further reduced to 20 ml for biotesting.

The aim of this study was not the exact analysis of specific compounds in sediments but a bioassay-directed screening of dominant toxicants. Therefore, extraction and fractionation methods were not optimized for specific compounds, and no recovery rates were determined. Concentrations of toxicants in sediments as given in this paper are approximate values without considering losses during the fractionation procedure.

Fractionation

The acetonic extract was fractionated in a two-step procedure beginning with a primary fractionation using column chromatography. If primary fractions were toxic and still too complex for toxicant identification and confirmation, a secondary fractionation step using normal-phase HPLC was applied. Fractions are marked as Fi.j with i signifying the fraction number in the primary fractionation step, and j representing the fraction number in the secondary step.

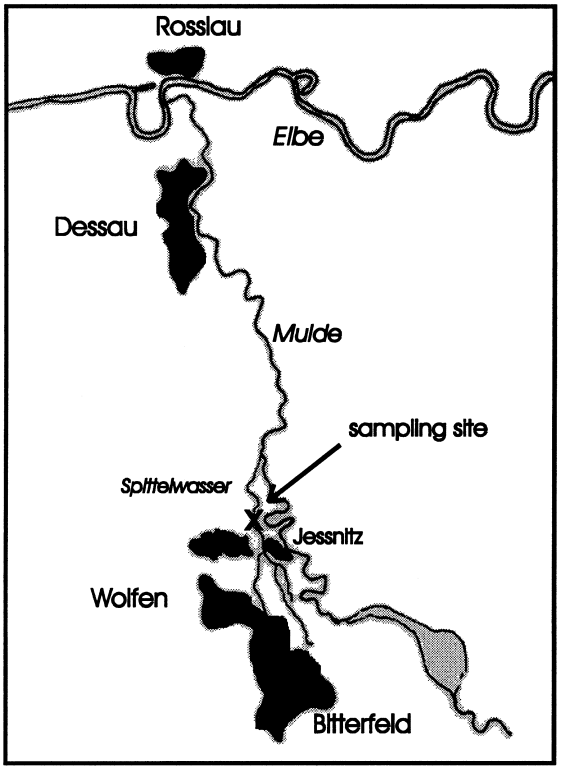


Fig. 1. Location of the sampling site

Table 1. Ignition loss and grain size composition of sediment samples

	%
Ignition loss	15
Fine sand (0.063–0.2 mm)	53.8
Coarse silt (0.02–0.063 mm)	14.6
Middle silt (0.0063–0.02 mm)	35.3
Fine silt (0.002–0.0063 mm)	2.2
Clay (<0.0063 mm)	7.7
Sum	113

Column Chromatography: For the fractionation, a second aliquot of 200 ml was adsorbed to alumina N (activity I, 10 g, specific surface 150 m^2/g, ICN Biomedicals GmbH, Eschwege, Germany) deactivated with 6% of water by solvent evaporation under reduced pressure and then applied to the top of fresh alumina (35 g) contained in a glass column (∅ 3 cm). Compounds were eluted with solvents of increasing polarity separating visible bands. F1, a colorless fraction was eluted with 70 ml n-hexane, followed by the slightly brown-colored fraction F2 eluted with 60 ml n-hexane. F3, the fraction between F2 and a region with strong red color was eluted with 2,000 ml n-hexane. The red-colored fraction F4 was eluted with 60 ml n-hexane/dichloromethane 50/50 followed by F5, representing a dark-brown fraction eluted with 200 ml dichloromethane/acetone 50/50. F6 was eluted with 200 ml methanol. All fractions were evaporated to dryness and redissolved in 20 ml acetone.

HPLC: The fractions F1 and F3 were subjected to a secondary fractionation using normal phase HPLC (Merck, Hitachi L-6250, Darmstadt, Germany) equipped with a silica gel column (Merck Si60, 25 · 250 mm, grain size 7 µm) and UV-VIS detection (Merck L-4250). UV-active compounds were detected at 250 nm. Secondary fractions were collected based on the peaks in the UV-chromatogramms. Injection volume was 5 ml.

Fraction F4 contained only a limited number of major compounds. Therefore a further fractionation was not necessary. The toxicity of F6 was small. Therefore, further investigation was renounced.

F1 was further fractionated using 10 ml/min n-hexane/tetrahydrofurane 97/3 as mobile phase. Eight secondary fractions were collected: F1.1 (0–10.5 min), F1.2 (10.5–12.2 min), F1.3 (12.2–12.7 min), F1.4 (12.7–13.3 min), F1.5 (13.3–13.7 min), F1.6 (13.7–15 min), F1.7 (15–16.2 min), and F1.8 (16.2–20 min).

For secondary fractionation of F3, a 30-min step-gradient was performed using a flow of 10 ml/min. The gradient started at n-hexane/tetrahydrofurane 97/3 which was held for 5 min, followed by a 10-min gradient to n-hexane/tetrahydrofurane 50/50, which was held for additional 15 min. Six secondary fractions were collected: F3.1 (0–17 min), F3.2 (17–19 min), F3.3 (19–20.8 min), F3.4 (20.8–22 min), F3.5 (22–23.4 min), and F3.6 (23.4–27 min). All secondary fractions were evaporated to dryness and dissolved in 5 ml acetone.

Removal of Sulfur: The contribution of elemental sulfur to toxicity was estimated removing sulfur with copper powder (Jacobs *et al.* 1992) and testing fractions before and after removal. Aliquots (5 ml) of the respective subfractions (F1.2 and F2.1) were shaken for 30 min with 1 g copper powder (99%, Baker, Deventer, The Netherlands) and filtered through glass fiber filters (GF/F, Whatman, Maidstone, England). Removal of sulfur was confirmed with GC/MSD.

GC-MS Analysis

The components of the fractions were separated by gas chromatography (model 5890 II, Hewlett Packard, Waldbronn, Germany) on a 5% diphenyl dimethyl polysiloxane capillary (model HP-5MS, Hewlett Packard; 30 m · 0.25 mm, 0.25 µm film thickness) with helium as carrier gas using a column head pressure of 10 hPa. Aliquots of 1 µl acetonic solution were injected splitless. The injector temperature was 280°C. The column temperature was held at 70°C for 4 min and then increased with a rate of 7°/min to 280°C and held for 20 min. For the identification of unknown compounds, the mass spectrometer (model 5971, Hewlett Packard; 70 eV, 280°C) was scanned from m/z 50 to 500. Compounds were identified using a spectra library (NIST/EPA/NIH Mass Spectral Library, National Institute of Standards and Technology, USA). Compounds expected to cause observed toxicity were confirmed comparing spectra and retention time with standard compounds. Quantification was done in selected-ion mode using external standards.

Biotests

Acetonic fractions were applied to the biotests at a maximum concentration of 1%, which proved to be nontoxic to all biotests, and at pH values as recommended for the respective biotest. Solubility of the components of the acetonic fractions in water remained incomplete for most fractions. Precipitates were allowed to settle down and were not further considered. In the case of growth inhibition test with algae additional filtration through glass fiber filters (GF/F, Whatman) was necessary to eliminate particles disturbing cell counting.

Toxicity to *Vibrio fischeri*, *Daphnia magna*, and *Scenedesmus vacuolatus* is expressed as effect dilution ($ED50_A$) following the nomenclature of the International Standardisation Organisation (ISO 5667-16 1998) who introduced the term dilution suggesting LID (lowest ineffective dilution) values for the effects of environmental samples. $ED50_A$ values, which can be determined with a higher precision than LID values, are defined as the dilution of the acetonic extract, of a fraction ($ED50_{AF}$), or of a solution of a mixture of standard compounds ($ED50_{AM}$) in acetone at which a 50% reduction of luminescence, survival, or cell multiplication is achieved, respectively (Equation 1).

$$ED50_A = (V_T/V_A)_{50\%\ solution} \quad \text{(Eqn. 1)}$$

V_T and V_A are the test volume and the volume of the acetonic solution, respectively. $ED50_A$ values were calculated using the PROBIT model (SAS Institute Inc., Cary, NC). To achieve comparability with other sediments, all dilution factors were transformed to the theoretical amount of sediment in g which has to be extracted and applied in the biotests per liter medium to achieve 50% inhibition ($ED50_S$) (Equation 2).

$$ED50_S = ED50_A^{-1} * (m_S/V_E) \quad \text{(Eqn. 2)}$$

m_S and V_E are the mass of the extracted sediment (410 g) and the corresponding volume of the extract before fractionation (0.05 L). Losses during fractionation and incomplete extraction could not be quantified because of the large number of possible toxicants and are therefore not considered in the transformation. An ED50 value of 100 representing the 1% dilution of acetonic extract in distilled water corresponds to 82 g/L sediment, the highest concentration tested.

To visualize toxicity patterns, relative toxic potencies were calculated by dividing all $ED50_A$ values by the highest $ED50_A$ for each biotest and fractionation step.

For economical reasons, testing of procedural blanks, *e.g.*, blank solvent processed through the different fractionation steps, was renounced. Therefore, procedural artifacts, which might be responsible for minor parts of unidentified toxicity, cannot be completely excluded. However, the major part of toxicity could be traced back to distinct toxicants such as pesticides and PAHs in high concentrations. It may be assumed that these should not originate from analytical-grade solvents or fresh and neat alumium oxide, silica gel, or glass-fiber filters.

Bioluminescence of V. fischeri: The marine bacterium *V. fischeri* (NRRL-B-11177, Dr. Bruno Lange GmbH, Berlin, Germany) was used for testing the inhibition of bioluminescence, which is related to the energy metabolism of the organism. Bioluminescence was measured according to ISO 11348 (1998) in a saline medium (2% NaCl) at pH 7 and 15°C using the LUMIStox system (Dr. Bruno Lange GmbH). The incubation time was 30 min.

Immobilization of D. magna*:* According to DIN 38412 L 30 (1989) immobilization of 2- to 26-h old *Daphnia* (*D. magna*) was observed. Samples were tested at a pH of 8 in two replicates using 20-ml test vessels, each containing five organisms. Samples were diluted with oxygen saturated water. After 24 h of exposure, the number of immobile *Daphnia* was determined.

Algal Growth Inhibition Test with S. vacuolatus*:* Synchronous cultures of the unicellular green alga *S. vacuolatus* (strain 211-15, SAG, Göttingen, Germany) were used as a test system. The parameter of toxicity was the inhibition of the cellular reproduction during one generation cycle lasting 24 h at a pH of 7 according to the procedure described by Altenburger *et al.* (1990). Modifications were made regarding carbon supply at $NaHCO_3$ and utilization of gas-tight 50-ml test tubes to allow for testing of foaming and volatile substances. The cell number and the cell volume distribution were analyzed utilising a cell counter (CASY II, Schärfe System, Reutlingen, Germany).

Confirmation of Toxicants

For confirmation, expected toxicants were purchased as neat compounds, dissolved in acetone as mixtures corresponding to the composition, and the concentrations of the toxic fractions and tested for toxicity in the same way as the fractions. $ED50_{AF}$ values for the fractions and $ED50_{AM}$ values for the corresponding mixtures were calculated and compared. Toxicants are looked upon as confirmed if the ratio $ED50_{AM}$ / $ED50_{AF} \cdot 100\%$ is $100 \pm 40\%$. For comparison with data from literature, EC50 values were assessed using acetonic solutions of single compounds dividing their concentration by the effect dilution $ED50_A$.

Results

Effects of Sediment Extract

ED50 values of the primary acetonic extract of below 1 to 4.3 g sediment per liter test medium indicate high toxicity with *V. fischeri* being the most sensitive organism (Table 2).

Effects of Fractions

Relative toxic potencies as defined above and $ED50_S$ [g sediment/L] values of the most toxic fractions are shown in Figure 2. Toxicity of fractions shows significantly different patterns depending on the biotest. Fraction F1 was highly toxic to all of three biotests involved. This fraction was too complex for direct identification of toxicants. Thus, it was further fractionated, generating secondary fractions F1.1 to F1.8. From fraction F1 the toxicity to daphnids was recovered in F1.1, whereas toxicity to luminescent bacteria and algae was found in F1.2. In addition to F1.2, subfractions F1.6 to F1.8 exhibited high toxicity to green algae. Minor toxicity for all biotests was found in F2. Fraction F3 was highly toxic to algae and *Daphnia*, exhibiting only minor effects on luminescent bacteria. Like F1, fraction F3 was further fractionated, providing the secondary fractions F3.1 to F3.6. While F3.6 was the most toxic fraction to daphnids, toxicity to algae was quantitatively recovered in F3.4. High toxicity to algae was also found in fraction F4. Fraction F5 was slightly toxic to all biotests. F6 was the least toxic fraction.

The relative toxic potencies (Figure 2) are valid for the acetonic extract and fractions, but not for the native sediment, where they are modified by bioavailability.

Identification of Components of Toxic Fractions

Major toxic fractions identified in this study are F1, more specifically the toxic subfractions F1.1, F1.2, F1.6, F1.7, and F1.8; F3 with the subfractions F3.4 and F3.6; and F4 and F5. These fractions were selected for analytical identification and quantification of potential toxicants (Table 3). Further investigation of F2 was dispensed due to overlapping of constituents with F1. All fractions were screened with GC/MSD applying the spectra library in order to evaluate the fractionation procedure.

The column chromatographic fractionation on aluminium oxide (alumina N) provided six fractions containing compounds with increasing polarity. Properties determining chromatographic normal phase separations are the existence of polar substituents and molecular size and shape.

Table 2. Toxicity of acetonic extract to different organisms

Organism	$ED50_s$ (g sediment/L)
Vibrio fischeri	0.83
Daphnia magna	4.3
Scenedesmus vacuolatus	3.1

F1 and F2 contain nonpolar aliphatic and aromatic compounds such as tetrabutyltin, alkanes, alkenes, sulfur, biphenyls, and PAHs (Table 3). HCH isomers and DDT were only found in fraction 2. In fraction F3 typical components are aromatic esters, such as alkylsulfonic acid phenylesters, phthalates, the aromatic amine N-phenyl-β-naphthalene amine, the methoxy analogon of DDT methoxychlor and the phosphoric ester methyl parathion. Despite its similarity to DDT, methoxychlor is characterized by much stronger retention in normal-phase chromatographic systems because of the acidic character of the hydrogen substituents of methyl groups next to the oxygen atoms (Grimvall and Östmann 1994). Fraction F4 is characterized by the polar s-triazine prometryn and a red azo dye. Mass spectrum and suggested chemical structure is shown in Figure 3. Beside these compounds different phthalates and some nonidentified compounds were detected. One of the major components of fraction F5 is n-tributyltin with ionic properties when dissolved in water. The most polar fraction, F6, exhibited low toxicity and was not characterized analytically.

Though direct identification of constituents in fractions F4 and F5 was possible, the more complex fractions F1 and F3 demanded further fractionation. Normal-phase HPLC fractionation of F1 (Figure 2) resulted in fractions with increasing aromaticity beginning with aliphatic compounds in F1.1 without a significant UV absorption (Figure 2) followed by chloro- and alkyl-substituted mono- and diaromatic compounds in F1.2, which also contained elemental sulfur. Both fractions F1.1 and F1.2 contained a large number of compounds, the majority of which could be characterized as saturated or nonsaturated aliphatic hydrocarbons (F1.1) or polychlorinated or alkylated biphenyls, naphthalenes, and other mono- and diaromatic compounds (F1.2) without identification of the respective isomere. F1.2 generated the major peak in the UV-chromatogram (Figure 2). Nonsubstituted diaromatic compounds were found in fraction F1.3. Fractions F1.4 and F1.5 contain alkyl-substituted triaromatic PAHs and some larger diaromatic compounds such as stilbenes and 4,4′-dichloro-1,1′-diphenylmethane. The fractions F1.6 to F1.8 are characterized by nonsubstituted PAHs with an increasing number of aromatic rings. The major compounds were identified and quantified. HPLC fractionation of F3 provided secondary fractions containing aromatic esters such as alkylsulfonic acid phenylesters and/or phenoxyalkanes and phthalates (F3.1–F3.3) followed by the aromatic amine N-phenyl-β-naphthalene amine (F3.4) representing the major peak in the UV chromatogram, the more polar methoxy analoga of DDT and DDE (F3.5), and the phosphoric ester methyl parathion (F3.6). Additional nonidentified peaks were detected in all subfractions.

Within the different fractions several unusual, not frequently analyzed compounds have been detected, such as 4,4′-dichlorodiphenylsulfide, 4,4′-dichloro-1,1′-diphenylmethane (a substitute for PCBs in transformers and hydraulics), and alkylsulfonic acid phenylesters, which are used as softening

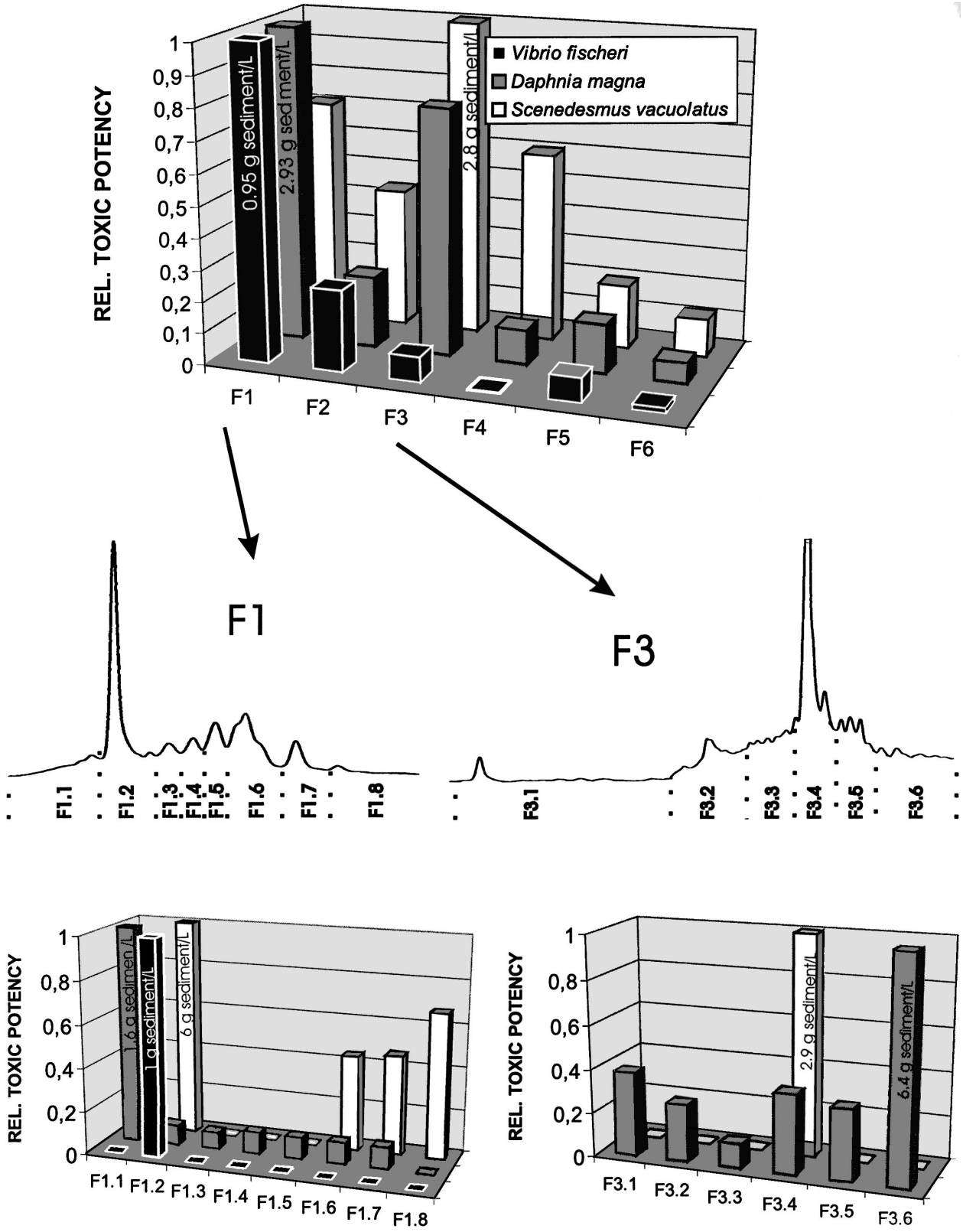


Fig. 2. Relative toxic potencies of primary and secondary fractions as well as $ED50_S$ values in g sediment/L for the most toxic fractions. HPLC chromatograms (at 250 nm) and fractionation scheme for F1 and F3 are shown. The dashed lines in the HPLC chromatograms indicate separation points for the fractions

Table 3. Major compounds identified in fractions of sediment extract. Constituents of selected toxic fractions in bold letters were identified and quantified using external standards. The other ones were identified using spectra library

Fraction	Characterization	Identified Major Compounds
F1.1	Aliphatic compounds	**n-tetrabutyltin, n-alkanes C11–C29,** alkenes
F1.2	Sulfur, mono- and diaromatic compounds	sulfur, 4,4′-dichlorodiphenylsulphide, methyl- and ethyl-biphenyls, dichloronaphthalene, heptachlorostyrene, PCBs
F1.3	Diaromatic compounds	diphenylether, naphthalene, biphenyl
F1.4	Diaromatic and triaromatic compounds with aliphatic substituents	methylstilbenes, ethenyl-anthracene, dimethyl-phenanthrenes
F1.5	Diaromatic and triaromatic compounds with aliphatic substituents	stilbene, 4,4′dichloro-1,1′-diphenylmethane, 1-methyl-anthracene, 2-methyl-anthracene, phenyl-indenes, phenylmethyl-naphthalene
F1.6	Triaromatic compounds	**phenanthrene, anthracene, 2-phenylnaphthalene**
F1.7	Polyaromatic compounds (4 rings)	**fluoranthene, pyrene**
F1.8	Polyaromatic compounds (4–5 rings)	**benzo[ghi]fluoranthene, triphenylene, benz[a]anthracene, 2-ethylhexyl-phthalate**
F2	Nonpolar compounds	see F1, additional HCH, DDT, DDD
F3.1	Alkylsulfonic acid phenylesters and/or phenoxyalkanes, phthalates	diisooctylphthalate
F3.2		butyl-2-methylpropylphthalate
F3.3		
F3.4	Aromatic amine	**N-phenyl-β-naphthalene amine**
F3.5	Methoxy analogs of DDT and DDD	methoxychlor, 2,2-dichloro-1,1-bis (4-methoxyphenyl) ethane
F3.6	Phosphoric ester	**methyl parathion**
F4	s-Triazine, azo-dye	**prometryn,** azo-dye
F5	Ionizable organotin compound	**n-tributyltin**

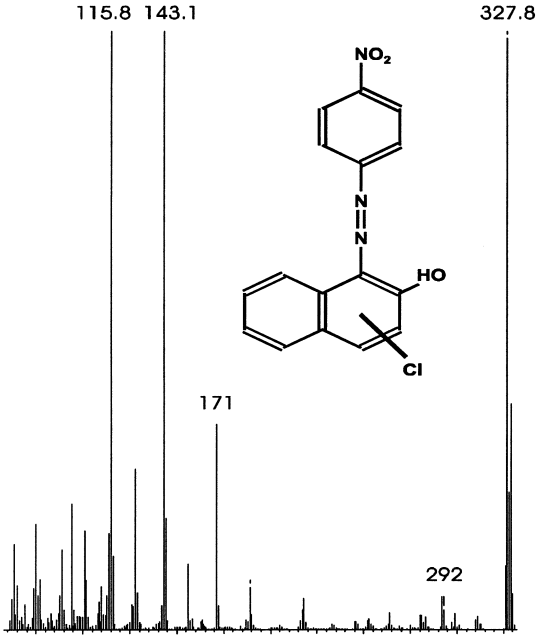


Fig. 3. Spectra and suspected formula of the azo-dye in fraction F4

agents for polyvinyl chloride. These compounds have been also identified in an earlier study by Franke *et al.* (1996) in sediments of the River Mulde. Alkysulfonic acid phenylesters and phenoxyalkanes with the basic peak at m/z 94 and no other characteristic peaks could not be distinguished.

Confirmation of Toxicants

To confirm identified compounds as toxicants causing the measured effects in the fractions, compounds were quantified and applied to the respective biotests as mixtures or single compounds dissolved in acetone in concentrations as measured in the fraction. $ED50_A$ values of these solutions were compared with those determined with the fractions (Table 4). In the case of sulfur toxicity, an indirect confirmation was applied removing sulfur with copper powder and subsequent retesting (Table 5).

Toxicity of fraction F1 was recovered in subfractions F1.1 (*Daphnia*), F1.2 (bacteria and algae), and F1.6 to F1.8 (algae). Major components of F1.1 are n-alkanes and n-tetrabutyltin. However, they were found to be responsible for only about one fourth of the toxicity at the concentration being present in this fraction (Table 4, $ED50_{AM}$ / $ED50_{AF}$) of the fraction. The major part of toxicity is obviously caused by compounds not yet identified. Possible toxicants contributing to the effect are aliphatic nonsaturated and cyclic compounds of which numerous have been detected in fraction F1.1. Because of the great number of possible isomers an identification of single compounds was not possible.

Toxicity of F1.2 to bacteria and algae was suspected to be caused by elemental sulfur. This holds for luminescent bacteria, but not for algae (Table 5). While toxicity to *V. fischeri* completely disappeared after removal of sulfur, toxicity to *S. vacuolatus* was not altered. Therefore, the high toxic response of bacteria towards F1.2 can be traced back to sulfur. Toxicity to algae is likely to be caused by diaromatic compounds present in F1.2. Because of the great number of possible compounds and the fact that a lot of the components are commercially not available, an unambiguous identification and confirmation was not possible. Mixtures of phenanthrene, anthracene and 2-phenyl-naphthalene, of fluoranthene and pyrene and of benzo-[ghi]fluoranthene, benz[a]anthracene, triphenylene, and 2-ethylhexylphthalate were confirmed as the cause for toxic effects of subfractions 1.6 to 1.8 to *S. vacuolatus* with a correspondence of 68%, 99%, and 92%, respectively (Table 4, $ED50_{AM}$ / $ED50_{AF}$).

Table 4. Confirmation of toxicants. $ED50_A$ values of mixtures of standard compounds ($ED50_{AM}$) and of fractions ($ED50_{AF}$) are given in dilution factors. Concentrations are given in mg/kg sediment ($conc_s$) and mg/L extract ($conc_A$). A dilution factor of 100 corresponds to 82 g sediment/L and is the lowest testable dilution

Fraction	Biotest	Toxicants	$Conc_s$ (mg/kg sediment)	$Conc_A$ (mg/L extract)	$ED50_{AF}$ (dilution)	$ED50_{AM}$ (dilution)	$ED50_{AM}$/ $ED50_{AF}$ (%)
F1.1	*Daphnia*	n-alkanes:			2,100	475	23
		C13	5.56	45.6			
		C14	18.8	154			
		C15	46.5	381			
		C16	48.3	396			
		C17	32.6	267			
		C18	16.6	136			
F1.1	*Daphnia*	n-tetrabutyltin	5.37	44	2,100	100	5
F1.6	*Scenedesmus*	phenanthrene	5.9	48.1	588	400	68
		anthracene	1.1	8.9			
		2-phenyl-naphthalene	5.0	41.0			
F1.7	*Scenedesmus*	fluoranthene	3.83	31.4	625	617	99
		pyrene	7.60	62.3			
F1.8	*Scenedesmus*	benzo[ghi]fluoranthene	2.47	20	909	833	92
		benz[a]anthracene	0.35	2.8			
		triphenylene	0.60	4.9			
		2-ethylhexyl-phthalate	2.10	17			
F3.4	*Scenedesmus*	N-phenyl-β-naphthalene	15.8	130	2,778	6,842	246
F3.6	*Daphnia*	methyl parathion	1.6	13	1,310	1,270	97
F4	*Scenedesmus*	prometryn	6.34	52	1,370	1,723	126
F5	*Vibrio*	n-tributyltin	2.44	50.0*	1,663	2,610	157
	Daphnia	n-tributyltin			633	6,080	960
	Scenedesmus	n-tributyltin			1,493	980	66

* Fraction F5 was 2.5-fold concentrated compared to the other fractions

Table 5. Indirect confirmation of sulfur toxicity by removal. $ED50_A$ values are given in dilution factors. A dilution factor of 100 corresponds to 82 g sediment/L and is the lowest testable dilution

Fraction	Biotest	$ED50_A$ (dilution)	$ED50_A$ After Sulfur Removal (dilution)
F1.2	*Vibrio*	3,500	<100
F1.2	*Scenedesmus*	1,370	1,149

Toxicity of fraction F3.4 to algae could be traced back to N-phenyl-β-naphthalene amine. ED50 of a solution of this compound containing the same concentration as the fraction was 2.5-fold higher than the ED50 of the fraction. Matrix effects or antagonistic interactions with unidentified compounds appear to reduce toxicity of the compound in the fraction. Methyl parathion was confirmed as the toxicant causing the effect of fraction F3.6 to daphnia with a correspondence of 97%.

In fraction F4 the herbicide prometryn was confirmed as the principal toxicant causing the effect to algae. The correspondance of 126% is well within the uncertainities of the measurements. n-Tributyltin was confirmed as the major toxicant in fraction F5 to all of the biotests. However, a reduction of toxicity of n-tributyltin by factors of 1.5 and 9.6 to daphnids and bacteria, respectively, has been observed when the compound was applied as a part of the fraction compared to the standard solution. Dissolution of F5 in water resulted in strong precipitation of nonsoluble organic compounds, potentially reducing bioavailability of n-tributyltin by adsorption. Sorption to precipitated solids or antagonistic effects with nonidentified compounds thus appears to be responsible for the reduced toxicity of n-tributyltin in fraction F5. If this also holds for the toxicity of F5 to algae, the correspondence of 66% might overestimate the contribution of n-tributyltin to algal toxicity. This suggests that unidentified toxicants might play a significant role for algal toxicity of F5.

The sum of $ED50_A$ values of the fractions F1 to F6 exceeds the $ED50_A$ value of the whole extract by a factor of about three for *D. magna* and *S. vacuolatus* (Table 6). For both organisms, significant toxicity is spread over several fractions. In contrast to that, the toxicity of the extract to *V. fischeri* is dominated by only one fraction (F1) showing a good correspondence between the toxicity of the extract and the additive toxicity of the fractions. Similar observations can be made with the subfractions of F1 and F3 (Table 7, Table 8). There is a good correspondence for the toxicity of those fractions, which is clearly dominated by the toxicity of one subfraction (F1.2 with *V. fischeri* and F3.4 with *S. vacuolatus*). For the other tests the additive toxicity of the subfractions exceeds the toxicity of the fraction. The model of concentration additivity (Brown 1968) is generally accepted for similar acting compounds. Dissimilar toxicants are expected to act according to the model of independent combination effects. The effect of the mixture is lower than expected from the model of concentration additivity, but higher than the effect of the most potent compound alone (Grimme *et al.* 1996). In contrast to the compounds within one subfraction with rather similar chemical properties, complex fractions containing compounds with rather dissimilar compounds cannot be expected to act in a similar way. Because of the complexity of the fractions similar and dissimilar action is

Table 6. Relation of the $ED50_A$ value of the whole extract and the sum of $ED50_A$ values of the fractions F1 to F6 ($\Sigma_{i=F1}^{F6} ED50_{Ai}$)

	$ED50_A$	$\Sigma_{i=F1}^{F6} ED50_{Ai}$	$\Sigma_{i=F1}^{F6} ED50_{Ai}$/ $ED50_A$ (%)
Vibrio fischeri	9,880	12,200	124
Daphnia magna	1,910	6,693	350
Scenedesmus vacuolatus	2,650	8,990	339

Table 7. Relation of the $ED50_A$ value of the fraction F1 and the sum of $ED50_A$ values of the subfractions F1.1 to F1.8 ($\Sigma_{i=F1.1}^{F1.8} ED50_{Ai}$)

	$ED50_A$	$\Sigma_{i=F1.1}^{F1.8} ED50_{Ai}$	$\Sigma_{i=F1.1}^{F1.8} ED50_{Ai}$/ $ED50_A$ (%)
Vibrio fischeri	8,630	8,200	95
Daphnia magna	2,800	4,930	176
Scenedesmus vacuolatus	2,080	4,000	193

Table 8. Relation of the $ED50_A$ value of the fraction F3 and the sum of $ED50_A$ values of the subfractions 3.1 to F3.6 ($\Sigma_{i=F3.1}^{F3.6} ED50_{Ai}$)

	$ED50_A$	$\Sigma_{i=F3.1}^{F3.6} ED50_{Ai}$	$\Sigma_{i=F3.1}^{F3.6} ED50_{Ai}$/ $ED50_A$ (%)
Daphnia magna	2,170	2,650	122
Scenedesmus vacuolatus	2,929	2,778	95

likely to occur, resulting in combination toxicity being higher than expected if the model of independent action is taken as a basis, but lower than expected from the model of concentration additivity. The presented data are in good agreement with this consideration.

Discussion

Several pollutants in concentrations exhibiting acute toxic effects could be detected in acetonic extracts of Spittelwasser sediment.

The high toxicity of the sediment extract to luminescent bacteria *V. fischeri* is caused by elemental sulfur, which was found in fractions F1 (high amounts) and F2 (lower amounts, data not shown). The high toxicity of the naturally occurring compound elemental sulfur to *V. fischeri* with an EC50 value of 24.7–35.8 µg/L is well known (Jacobs *et al.* 1992). It has been shown that in biotests with *V. fischeri* elemental sulfur may be not only the major toxicant in organic extracts of sediments (Jacobs *et al.* 1992; Salizzato *et al.* 1997; Svenson *et al.* 1998) but also if solid phase tests are applied to sediments (Salizzato and Pavoni 1998).

n-Alkanes with chain length from 13 to 18 were the major components of F1.1, a fraction that was found to be particularly toxic to daphnids. The alkanes appear to be responsible for about one-fourth of the toxicity of the fraction. Toxicity data for this group of chemicals are scarce, although they are typical constituents of petroleum oils of which about 650 tons per year are transported to the North Sea via the river Elbe (Theobald *et al.* 1995). Oxidative biodegradation is rather fast with a half-life time for n-pentadecane and n-hexadecane of about 3 weeks (Verschueren 1996). Degradation in anaerobic sediments is expected to be much slower.

n-Tetrabutyltin contributing with about 5% to toxicity of F1.1 is a well-known contaminant of the Rivers Spittelwasser, Mulde, and Elbe. This compound has been emitted together with n-tributyltin by a butyltin plant at Bitterfeld (Kuballa *et al.* 1995). Toxicity data for n-tetrabutyltin are rare. Existing data suggest a much lower toxicity and bioavailability than for n-tributyltin. However, in the environment n-tetrabutyltin may be degraded by debutylation to n-tributyltin (Boopathy and Daniels 1991; Kuballa *et al.* 1995).

n-Tributyltin was found to be the major toxicant in fraction F5 affecting luminescent bacteria and algae. n-Tributyltin, used as antifouling agents in paints for boats, ships, and docks, is probably the most toxic agent to aquatic organisms that has ever been introduced deliberately into aquatic environments. EC50 of n-tributyltin for *V. fischeri* was determined by Bundy *et al.* (1997) with 0.022 mg/L corresponding well with the value 0.019 mg/L ($Conc_A$ / $ED50_{AM}$, Table 4) estimated in this study. For algae, only toxicity data for n-tributyltin oxide exist with EC50 values of <0.02 to 5 µg Sn/L (Maguire 1987; Fargasova and Kizlink 1996). However, this extremely high toxicity could not be confirmed for n-tributyltin chloride. For this compound in the present study, an EC50 value ($Conc_A$ / $ED50_{AM}$, Table 4) of 51 µg/L was measured. In the Spittelwasser sediment the presence of n-tributyltin is obviously due to emissions from production processes because there is no boat traffic on the River Spittelwasser. In an earlier study of sediments of Spittelwasser 7 mg/kg tributyltin have been detected (Kuballa *et al.* 1995). The concentrations found in this investigation were about 2.6-fold lower. This suggests a reduction of n-tributyltin concentrations since the sampling by Kuballa *et al.* (1995) shortly after closedown of butyltin production. For the reduction of n-tributyltin concentrations in sediments two major pathways can be expected: degradation and desorption. N-Tributyltin was reported to be degraded by 50% in soils and sediments under aerobic conditions within 100 to 140 days (Maguire and Tkacz 1985; Maguire 1987). However, degradation in anaerobic soils and sediments like Spittelwasser appears to be much slower with half-lives in the range of years (Fent and Hunn 1995). In sediments of the harbor of Toronto a sediment-water partitioning coefficient of 2,180 has been measured. The rate of desorption was strongly dependent on agitation (Maguire and Tkacz 1985). In Swiss harbors concentrations in the sediments were enhanced by a factor of 1,000 compared with concentrations in the water column. Estimated sorption coefficients for suspended particulates were in the range of 1,500 to 27,000 (Fent and Hunn 1995). If equilibrium between pore water and sediment is assumed and the concentration of 2.44 mg/kg sediment and a partitioning coefficient in the range of 1,500 to 27,000 is taken as a basis, a pore water concentration of about 0.1 to 1.6 µg/L n-tributyltin can be estimated. This is in the range of effect concentrations of sensitive organisms reported in literature (<0.05 to 24 µg/L; Maguire 1987). If an uptake of the compound with ingested contaminated particles is assumed, an increased hazard must be expected. Direct uptake from the sediment by oligochaetes has been reported by Maguire and Tkacz (1985), who suggested an introduction of the compound in the aquatic foodweb by fish feeding on benthic organisms.

The phosphorous insecticide methyl parathion, which is known as an inhibitor of acetylcholinesterase (Aldridge and Reiner 1972) could be identified and confirmed as the toxicant

being responsible for the high toxicity of fraction F3.6 to daphnids. It is present in high concentrations of around 1.6 mg/kg in the Spittelwasser sediment, thus exceeding significantly concentrations in comparable sediments like that of a drain of waste water from a methyl parathion manufacturing plant in Egypt with about 90 µg/kg (Abd-Allah 1995). Note, however, that in the present study only fine sediment (grain size <0.2 mm) was investigated, which is expected to be the major sorbent.

Methyl parathion has been produced in Bitterfeld up to 1991 (Chemie AG Bitterfeld-Wolfen 1993). Despite its high chemical and biological degradation rate (Ferrando *et al.* 1992; Megharaj *et al.* 1994) with a half-life of 11 h (Ferrando *et al.* 1992) to 30 days (Pritchard *et al.* 1987) in natural waters, our present results show that it is still present in ecotoxicologically relevant concentrations in the Spittelwasser sediment. This suggests ongoing delivery from abandoned waste dumps in Bitterfeld or stabilization of adsorbed methyl parathion under anaerobic conditions. High content of organic matter in the sediment (grain size <0.2 mm) with an ignition loss of 15% is expected to result in relatively high sorption capacity. In the River Spittelwasser residues of production of the ion exchanger wofatit (Chemie AG Bitterfeld-Wolfen 1993) have also been discharged, which may contribute to the sorption and apparent persistence of xenobiotics like methyl parathion. Interestingly, high concentrations of methyl parathion in Mediterranean sediments diminished to about 1% within 2 months (Badawy *et al.* 1984).

Methyl parathion is highly toxic to aquatic invertebrates. Pickering *et al.* (1989) reported an EC50 of 12 µg/L for *D. magna*, which is in good agreement with the EC50 value ($Conc_A$ / $ED50_{AM}$, Table 4) determined in this study (10 µg/L). The EC50 of methyl parathion to benthic midge larvae *Chironimus riparius* determined with spiked sediment containing 3% organic carbon was 4 µg/kg (Fisher *et al.* 1993). Concentrations in the Spittelwasser sediment (grain size <0.2 mm) containing 1.6 mg/kg are about 400-fold higher (Table 8). The EC50 of methyl parathion has been about 2.6-fold lower if *C. riparius* has not been exposed to spiked sediment but to an acetonic solution of methyl parathion in a water-only experiment (Fisher *et al.* 1993). This relatively low value suggests a high bioavailability of the compound, which is in qualitative agreement with the moderate log K_{OW} of 3.32 (Fisher *et al.* 1993) and a water solubility of 55 to 60 mg/L (Verschueren 1996). In a former TIE study in agricultural drain water from American rice fields methyl parathion has been already identified as a toxicant causing severe effects to wildlife (Finlayson *et al.* 1993).

The high toxicity of F3 toward the unicellular algae *S. vacuolatus* was caused by N-phenyl-β-naphthalene amine in the secondary fraction F3.4. This compound is used as a rubber antioxidant with an occurrence of up to 1% in finished rubber (Verschueren 1996). Data about algal toxicity are scarce. The EC50 ($Conc_A$ / $ED50_{AM}$, Table 4) measured in this study is 19 µg/L, which is in the range of s-triazine herbicides (Gaggi *et al.* 1995). N-phenyl-β-naphthalene amine is known to occur as a natural product of water hyacinth (*Eichornia crassipes*) with an allelopathic effect on algae (Yang *et al.* 1992). The toxicity to the green algae *Chlamydomonas reinhardtii* is about 2.5 times higher than the toxicity of $CuSO_4$ (Sun *et al.* 1993). Toxicity to the fish species *Oryzias latipes* is about 40-fold lower than to green algae (Yoshioka and Ose 1993). These findings suggest that N-phenyl-β-naphthalene amine acts specifically as a phytotoxin, possibly having a substantial impact on primary producers in natural waters even at quite low concentrations. Interestingly, this compound has been analyzed in the environment only rarely (for example, Okumura 1995) and has not been considered in the context of hazard assessment.

In fraction F4, the s-triazine prometryn that was produced in Bitterfeld from 1960 to 1990 (Chemie AG Bitterfeld-Wolfen 1993), was confirmed as the chemical causing significant toxicity to algae. s-Triazines are known to inhibit photosynthetic electron transport (van Rensen 1982), and EC50 values of 21 and 53 µg/L have been reported for prometryn and the green algae *Selenastrum capricornutum* and *Dunaliella tertiolecta*, respectively (Gaggi *et al.* 1995). The EC50 value ($Conc_A$ / $ED50_{AM}$) of 30 µg/L for *S. vacuolatus* corresponding to the ED50 value (Table 4) measured in this study fits well to these data. The EC50 for *V. fischeri* was not measurable, being well above the reported water solubility of 37.7 mg/L (Gaggi *et al.* 1995). As for methyl parathion, it is remarkable that prometryn can still be detected in high and ecotoxicologically relevant concentrations in Spittelwasser sediment despite the stop of production 8 years ago and despite its physicochemical profile as characterized by a relatively high water solubility (33 mg/L; Tomlin 1994), moderate lipophilicity (log K_{OW} of 3.34; Gaggi *et al.* 1995) and a degradation half-life of 40 to 70 days (Turin and Bowman 1997). K_{OC} values range from 190 to 617 ml/g depending on the pH of the solution with enhanced ionization and enhanced sorption at lower pH (Turin and Bowman 1997). Assuming a content of 50% organic carbon in organic matter, the prometryn concentration of 6.34 mg/kg sediment (content of organic matter of 15%, Table 1) should lead to an equilibrium concentration of about 0.1 to 0.4 mg/L in water, which is three- to tenfold higher than the EC50 of green algae, indicating a significant toxic potential of the compound in aquatic ecosystems.

Mixtures of PAHs containing anthracene, phenanthrene, 2-phenylnaphthalene, fluoranthene, pyrene, benzo[ghi]fluoranthene, triphenylen, and benzo[a]anthracene inhibited the growth of green algae *S. vacuolatus* in concentrations of 10 to 100 µg/L. Toxicity data for PAHs with green algae in literature are scarce and only available for selected compounds. However, these data indicate a generally lower toxicity than measured in the present study. Verschueren (1996) reports an EC50 value for fluoranthene with *S. capricornutum* of 54 mg/L and for *Skeletonema costatum* of 45 mg/L, values which are far above the solubility in water (0.265 mg/L, Verschueren [1996]), and an EC50 for benzo[a]anthracene above its saturation concentration in water. It remains to be clarified whether high PAH toxicity in this study is caused by all constituents of the mixture in similar amounts or whether some PAHs exhibit specific effects. Moreover, further investigations are needed to clarify whether high toxicity to green algae is caused by the PAHs themselves or by products of photomodification, which may show significantly enhanced phytotoxicities as was reported for *Lemna gibba* by Huang *et al.* (1995) and Ren *et al.* (1994). Toxicity to green algae, therefore, might also depend on the light flux applied during the test. Photoinduced toxicity of PAHs was also reported for benthic invertebrates such as *Hyalella azteca* and *Lumbriculus variegatus* (Ankley *et al.* 1994). Note that the photoinduced enhancement of toxicity of PAHs bound to sediments will become ecotoxicologically

relevant when water depth decreases during dry seasons or when sediments are dredged. In addition to the enhanced toxic potency, photo-oxidized PAHs are more water soluble and therefore more bioavailable than nonmodified compounds (Huang *et al.* 1995).

Besides the contaminants mentioned above there are additional toxicants in ecotoxicologically relevant concentrations, which could be detected in the aliphatic fraction F1.1 and the diaromatic fraction F1.2 with high toxicity to daphnids and green algae, respectively. Those compounds could not been chemically identified with the presently used approach.

Conclusions

Extracts of sediments of the River Spittelwasser contain xenobiotics in high concentrations exhibiting toxicity to algae (*S. vacuolatus*), invertebrates (*D. magna*), and bacteria (*V. fischeri*). The toxicants can be expected to originate from former industrial production in Bitterfeld. Despite the closedown of most plants about 7 years ago, environmental concentrations of these compounds are still high. Considering literature data characterizing bioavailability such as log K_{OW}, K_{OC}, water solubility, as well as toxicity data derived from spiked sediments, some individual compounds were found in concentrations in the sediments that represent a significant ecotoxic potential for indigenous organisms. This particularly holds for the relatively polar and highly toxic compounds methyl parathion affecting benthic and pelagic invertebrates, the pesticide n-tributyltin, which is toxic to organisms of all trophic levels, the herbicide prometryn, and N-phenyl-β-naphthalene amine. Methyl parathion, prometryn and N-phenyl-β-naphthalene amine, which were identified as major toxicants in this study, are compounds that have not been considered in former analytical studies of Spittelwasser sediment. This proves the significance of biological testing and toxicity identification for hazard assessment of environmental samples.

Moreover, the present study demonstrates the role of sediments as a memory for pollutants discharged into the environment in the past. For risk assessment however, further investigations concerning the influence of *in situ* conditions, including pH and DOC content of pore water and adsorption properties of the sediment, on exposure and toxicity of the identified toxicants have to be performed.

Acknowledgments. We thank Ms. Christmann, Ms. Kayser, Ms. Ränker, and Ms. Sperreuter for technical assistance. *Daphnia* tests were done by Dr. Roth bioTEST, Biologisches Umweltlabor, Heinrich-Rau-Straße 39. 04249 Leipzig, Germany.

References

Abd-Allah AMA (1995) Determination of organophosphorus pesticides in sediment from Alexandria-coast, Egypt. Toxicol Environ Chem 48:177–182

Aldridge WN, Reiner E (1978) Enzyme inhibitors as substrates. Interactions of esterases with esters of organophosphorous and carbamic acids. Amsterdam, The Netherlands

Altenburger R, Bödeker W, Faust M, Grimme LH (1990) Evaluation of the isobologram method for the assessment of mixtures of chemicals. Combination of effect studies with pesticides in algal biotests. Ecotoxicol Environ Saf 20:98–114

Ankley GT, Collyard SA, Monson PD, Kosian PA (1994) Influence of ultraviolet light on the toxicity of sediments contaminated with polycyclic aromatic hydrocarbons. Environ Toxicol Chem 13:1791–1796

Athey LA, Thomas JM, Miller WE, Word JQ (1989) Evaluation of bioassays for designing sediment cleanup strategies at a wood treatment site. Environ Toxicol Chem 8:223–230

Badawy MI, El-Dib MA, Aly OA (1984) Spill of methyl parathion in the mediterranean sea: a case study at Port-Said, Egypt. Bull Environ Contam Toxicol 32:469–477

Boopathy R, Daniels L (1991) Pattern of organotin inhibition of methanogenic bacteria. Appl Environ Microbiol 57:1189–1193

Brown VM (1968) The calculation of the acute toxicity of mixtures of poisons to rainbow trout. Wat Res 2:723–733

Bundy JG, Wardell JL, Campbell CD, Killham K, Paton GI (1997) Application of bioluminescence-based microbial biosensors to the ecotoxicity assessment of organotins. Lett Appl Microbiol 25:353–358

Chemie AG Bitterfeld-Wolfen (1993) Bitterfelder Chronik. 100 Jahre Chemiestandort Bitterfeld-Wolfen. Vorstand der Chemie AG Bitterfeld-Wolfen, Bitterfeld-Wolfen

DIN (Deutsche Industrienorm, German Industrial Standard) 19683 Blatt 2 (1973) Bodenuntersuchungsverfahren im Landwirtschaftlichen Wasserbau. Physikalische Laboruntersuchungen. Bestimmung der Korngrößenzusammensetzung nach Vorbehandlung mit Natriumpyrophosphat. Beuth Vertrieb GmbH, Berlin

DIN (Deutsche Industrienorm, German Industrial Standard) 38412 Teil 30 (1989) Deutsche Einheitsverfahren zur Wasser- Abwasser- und Schlammuntersuchung. Testverfahren mit Wasserorganismen (Gruppe L). Bestimmung der nicht akut giftigen Wirkung von Abwasser gegenüber Daphnien über Verdünnungsstufen. Beuth Vertrieb GmbH, Berlin

Fargasova A, Kizlink J (1996) Effect of organotin compounds on the growth of the freshwater alga *Scenedesmus quadricauda.* Ecotoxicol Environ Saf 34:156–159

Fent K, Hunn J (1995) Organotins in freshwater harbors and rivers: temporal distribution, annual trends and fate. Environ Toxicol Chem 14:1123–1132

Fernández P, Grifoll M, Solanas AM, Bayona JM, Albaigés J (1992) Bioassay-directed chemical analysis of genotoxic components in coastal sediments. Environ Sci Technol 26:817–829

Ferrando MD, Alarcon V, Fernandez-Casalderry A, Gamon M, Andreu-Moliner E (1992) Persistance of some pesticides in the aquatic environment. Bull Environ Contam Toxicol 48:747–755

Finlayson BJ, Harrington JA, Fujimura R, Isaac G (1993) Identification of methyl parathion toxicity in Colusa Basin drain water. Environ Toxicol Chem 12:291–303

Fisher SW, Lydy MJ, Barger J, Landrum PF (1993) Quantitative structure-activity relationships for predicting the toxicity of pesticides in aquatic systems with sediment. Environ Toxicol Chem 12:1307–1318

Forbes TL, Forbes VE, Giessing A, Hansen R, Kure LK (1998) Relative role of pore water versus ingested sediment in bioavailability of organic contaminants in marine sediments. Environ Toxicol Chem 12:2453–2462

Franke S, Schwarzbauer J, Link M, Francke W (1996) Identifizierung dimerer PAK und anderer ungewöhnlicher Aromaten in Umweltproben. In: Stegmann R (ed) Neue Techniken der Bodenreinigung. Hamburger Berichte Abfallwirtschaft, vol. 10. Technische Universität Hamburg-Harburg, Hamburg, pp 149–168

Gaggi C, Sbrilli G, El Naby AMH, Bucci M, Duccini M, Bacci E (1995) Toxicity and hazard ranking of s-triazine herbicides using microtox, two green algal species and a marine crustacean. Environ Toxicol Chem 14:1065–1069

Grifoll M, Solanas AM, Bayona JM (1990) Characterisation of genotoxic components in sediments by mass spectrometric techniques combined with Salmonella/microsome test. Arch Environ Contam Toxicol 19:175–184

Grimme LH, Faust M, Boedeker W, Altenburger R (1996) Aquatic toxicity of chemical substances in combination: still a matter of controversy. Hum Ecol Risk Assess 2:426–433

Grimvall E, Östmann C (1994) Retention characteristics of some selected halogenated environmental pollutants in silica and bonded normal-phase liquid chromatography. J Chromatogr A 675:55–64

Harkey GA, Landrum PF, Klaine SJ (1994) Comparison of whole-sediment, elutriate and pore-water exposures for use in assessing sediment-associated organic contaminants in bioassays. Environ Toxicol Chem 13:1315–1329

Ho KTY, Quinn JG (1993) Physical and chemical parameters of sediment extraction and fractionation that influence toxicity, as evaluated by Microtox®. Environ Toxicol Chem 12:615–625

Huang X-D, Dixon DG, Greenberg BM (1995) Increased polycyclic aromatic hydrocarbon toxicity following their photomodification in natural sunlight: impacts on the duckweek *Lemna gibba* L. G-3. Ecotoxicol Environ Saf 32:194–200

ISO 11348-2 (1998) Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (luminescent bacteria test). International Organization for Standardization, Geneva, Switzerland

ISO 5667-16 (1998) Water quality sampling, part 16. Guidance on biotesting of samples. International Organization for Standardization, Geneva, Switzerland

Jacobs MW, Delfino JJ, Bitton G (1992) The toxicity of sulfur to microtox from acetonitrile extracts of contaminated sediments. Environ Toxicol Chem 11:1137–1143

Knezovich JP, Harrison FL, Wilhelm RG (1987) The bioavailability of sediment-sorbed organic chemicals: a review. Water, Air Soil Poll 32:233–245

Kuballa J, Wilken R-D, Jantzen E, Kwan KK, Chau YK (1995) Speciation and genotoxicity of butyltin compounds. Analyst 120:667–673

Maguire RJ (1987) Environmental aspects of tributyltin. Appl Organomet Chem 1:475–498

Maguire RJ, Tkacz RJ (1985) Degradation of the tri-n-butyltin species in water and sediment from Toronto harbor. J Agric Food Chem 33:947–953

Megharaj M, Madhavi DR, Sreenivasulu C, Umamaheswari A, Venkateswarlu K (1994) Biodegradation of methyl parathion by soil isolates of microalgae and cyanobacteria. Bull Environ Contam Toxicol 53:292–297

Mount DI (1989) Methods for aquatic toxicity identification evaluation. Phase III toxicity confirmation procedures. US Environmental Protection Agency, EPA/600/3-88/036, Washington, DC

Mount DI, Anderson-Carnahan L (1989) Methods for aquatic toxicity identification evaluations. Phase II toxicity identification procedures. US EPA, EPA/600/3-88/035, Washington, DC

Neumeister H, Ruske R (1995) Immissionsgeprägte Böden der Region Bitterfeld. Mittlgn Dtsch Bodenk Ges 77:339–372

Norberg-King TJ, Mount DI, Durhan EJ, Ankley GT, Burkhard LP, Amato JR, Lukasewycz MT, Schubauer-Berigan MK, Anderson-Carnahan L (eds) (1991) Methods for aquatic toxicity identification evaluations. Phase I toxicity characterization procedures, 2nd ed. US EPA, EPA/600/6-91/003, Washington, DC

Okumura T (1995) Simultaneous determination of trace levels of water contaminants by capillary gas chromatography/mass spectrometry. J Environ Chem 5:597–604

Pickering Q, Carle DO, Pilli A, Willingham T, Lazorchak JM (1989) Effects of pollution on freshwater organisms. J WPCF 61:998–1042

Popp P, Kalbitz K, Oppermann G (1994) Application of solid-phase microextraction and gas chromatography with electron-capture and mass spectrometric detection for the determination of hexachlorocyclohexanes in soil solutions. J Chromatogr A 687:133–140

Pritchard PH, Cripe CR, Walker WW, Spain JC, Bourquin AW (1987) Biotic and abiotic degradation rates of methyl parathion in freshwater and estuarine water and sediment samples. Chemosphere 16:1509–1520

Ren L, Huang X-D, McConkey BJ, Dixon DG, Greenberg BM (1994) Photoinduced toxicity of three polycyclic aromatic hydrocarbons (fluoranthene, pyrene, and naphthalene) to the duckweed *Lemna gibba* L. G-3. Ecotox Environ Saf 28:160–171

Salizzato M, Pavoni B (1998) Sediment toxicity measured using *Vibrio fischeri* as related to the concentrations of organic (PCBs, PAHs) and inorganic (metals, sulphur) pollutants. Chemosphere 36:2949–2968

Salizzato M, Rigoni M, Pavoni B, Volpi Ghirardini A, Ghetti PF (1997) Separation and quantification of organic micropollutants (PAHs, PCB) in sediments. Toxicity of extracts towards *Vibrio fischeri*. Toxicol Environ Chem 60:183–200

Samoiloff MR, Bell J, Birkholz DA, Webster GRB, Arnott EG, Pulak R, Madrid A (1983) Combined bioassay-chemical fractionation scheme for the determination and ranking of toxic chemicals in sediments. Environ Sci Technol 17:329–334

Schutzle D, Lewtas J (1986) Bioassay-directed chemical analysis in environmental research. Environ Sci Technol 58:1060A–1075A

Sun F, Littlejohn D, Gibson MD (1998) Ultrasonic extraction and solid phase extraction clean-up for determination of US EPA 16 priority pollutant polycyclic aromatic hydrocarbons in soils by reversed-phase liquid chromatography with ultraviolet absorption detection. Anal Chim Acta 364:1–11

Sun WH, Yu SW, Yang SY, Zhao PW, Yu ZW, Wu HM, Huang SY, Tang CS (1993) Allelochemicals from root exudates of water hyacinth *Eichhornia crassipes*. Acta Phytophysiol Sinica 19:92–96

Svenson A, Viktor T, Remberger M (1998) Toxicity of elemental sulfur in sediments. Environ Toxicol Water Qual 13:217–224

Theobald N, Rave A, Jerzycki-Brandes K (1995) Input of hydrocarbons into the North Sea by the River Elbe. Fresenius J Anal Chem 353:83–87

Tomlin C (ed) (1994) The pesticide manual. Incorporating the agrochemicals handbook, 10th ed. Crop Publications, Cambridge, UK

Turin HJ, Bowman RS (1997) Sorption behavior and competition of bromacil, napropamide, and prometryn. J Environ Qual 26:1282–1287

van Rensen JJS (1982) Molecular mechanisms of herbicide action near photosystem II. Physiol Plant 54:515–521

Verschueren K (1996) Handbook of environmental data on organic chemicals. Van Nostrand Reinhold, New York, NY

Yang S, Yu Z, Sun W, Wu H (1992) Isolation and identification of antialgal compounds from root system of water hyacinth (*Eichhornia crassipes*). Acta Phytophysiol Sinica 18:399–402

Yoshioka Y, Ose Y (1993) A quantitative structure-activity relationship study and ecotoxicological risk quotient for the protection from chemical pollution. Environ Toxicol Water Qual 8:87–101

Kapitel III

Brauchen wir einen Biotest mit höheren Pflanzen in der aquatischen Toxikologie?

Beata Praszczyk, Rolf Altenburger, Jörg Oehlmann, Bernd Markert und Gerrit Schüürmann
In: J. Oehlmann, B. Markert (Hrsg.). *Ökotoxikologie. Ökosystemare Ansätze und Methoden.* Lansderg: ecomed. S. 151-163. (1999)

Brauchen wir einen Biotest mit höheren Pflanzen in der aquatischen Toxikologie?

B. Praszczyk[1], R. Altenburger[1], J. Oehlmann[2], B. Markert[2] und G. Schüürmann[1]

[1]Sektion Chemische Ökotoxikologie, UFZ-Umweltforschungszentrum Leipzig-Halle GmbH, Permoserstraße 15, 04318 Leipzig;

[2]Internationales Hochschulinstitut Zittau, Markt 23, 02763 Zittau.

veröffentlicht in:

J. Oehlman, B. Markert (Hrsg.). 1999. Ökotoxikologie. Ökosystemare Ansätze und Methoden. Landsberg: ecomed. S. 151-163.

Abstract

Bioteste mit aquatischen Organismen werden in den Bereichen der prospektiven Chemikalienbeurteilung und in der Umweltprobentestung verwendet. In der etablierten und normierten Biotestpalette befinden sich Testsysteme, die verschiedene Trophiestufen repräsentieren (Algen, Daphnien, Fische und Bakterien). International wird derzeit diskutiert, ob die einzelligen und systematisch als niedere Pflanzen zu betrachtenden Algen hinreichende Repräsentanten der autotrophen Lebensweise darstellen. In dieser Arbeit wurden daher die Sensitivität und Spezifität eines Biotestes mit der Wasserlinse *Lemna minor* untersucht.

Unsere Befunde zeigen, daß bei der Etablierung eines Biotestes mit einer höheren Wasserpflanze insbesondere die Wirkungsparameter und Testzeitpunkte reflektiert werden müssen. Zur Frage der Sensitivität und Spezifität des Lemnabiotestes wurden für ausgewählte herbizide Wirkstoffe Konzentrations-Wirkungs-Analysen durchgeführt. Der Lemnatest zeigt im Vergleich zu etablierten Algentesten bei verschiedenen Wirkprinzipien und Milieubedingungen deutlich höhere Sensitivitäten. Schließlich wurde das Ansprechverhalten von *L. minor* gegenüber Umweltproben für ein Längsprofil der Neiße studiert.

Auf Grundlage der ermittelten Ergebnisse stellt sich der untersuchte Biotest mit *Lemna minor* als eine sinnvolle Ergänzung zur Algenbiotestung dar.

Biotests using aquatic organisms are employed in the fields of prospective chemical hazard assessment and testing of effluents and other environmental samples. Conduct of established and standardised biotests comprises test systems that represent different trophic levels (algae, daphnids, fish and bacteria). In international fora there is currently a debate as to whether unicellular algae as systematically and strucuturally simple organisms are a satisfactory representation of all autotrophic life forms. This paper therefore studies the sensitivity and specificity of a biotest with the duckweed *Lemna minor*.

The results presented demonstrate that for the establishment of a biotest with a higher aquatic plant, effect parameter and toxicological endpoints require particular reflection. With respect to the question of sensitivity and specificity of responses concentration

response analysis was performed for selected herbicides. In comparison with well-established algal biotests *Lemna* shows significantly higher sensitivities for different herbicides with diverse modes of action. Finally the responsiveness to environmental samples was studied for a transect along the river Neiße.

On the basis of the findings there is a clear recommendation to regard the presented biotest using *Lemna minor* as a suitable supplementation to algal biotests.

1 Einleitung

Die Anwendungsgebiete biologischer Testverfahren sind vielseitig, von der prospektiven Bewertung der Umweltgefährlichkeit von Stoffen über die Feststellung von Gewässerverschmutzungen durch Einleitungen bis hin zu überwachendem Monitoring von Gewässern (Nusch 1992).

Im aquatischen Bereich gibt es vier regulativ geforderte Biotests für die Chemikalienbeurteilung: Leuchtbakterientest, Algentest, Daphnientest und Fischtest. Damit sollen verschiedene Trophiestufen und eine einfache Nahrungskette aus Produzenten, Primär- und Sekundärkonsumenten sowie Destruenten abgebildet werden.

Für die Standardisierung methodischer Details dieser Bioteste existieren deutsche Einheitsnormen (DIN) (Kanne 1989). Es wird zur Zeit über die Notwendigkeit einer Ergänzung dieser Biotestbatterie um ein Verfahren mit einer höheren Pflanze diskutiert (Greenberg et al. 1992, Fairchild et al. 1997, OECD 1997). Als geeignete Testorganismengruppe werden oft Lemnaceen vorgeschlagen (Lewis 1995). In der vorliegenden Arbeit werden Ergebnisse einer Biotestung mit der Wasserlinse *Lemna minor* mit Ergebnissen aus Tests mit einzelligen Algen verglichen.

Lemnaceen repräsentieren eine photoautotrophe Gruppe sehr stark reduzierter monokotyler Pflanzen (Augusten 1984). Sie sind die kleinsten Blütenpflanzen, die auf der Wasseroberfläche schwimmen. Die Familie der Lemnaceen umfaßt 4 Gattungen (*Lemna, Spirodela, Wolffia* und *Wolfiella*) mit 35 Spezies, die sich meist vegetativ durch Bildung von Tochterpflanzen (Fronds) vermehren. In Europa vorkommende Spezies sind: *Spirodella polyrhiza, Sp. punctata, Lemna trisula, L. minor, L. gibba, L. minuscula* und *Wolfia arrhiza*. Die einzelne Pflanze besteht aus einem Mutterfrond, aus dem links und rechts in Taschen Tochterfronds heranwachsen (Landolt und Kandeler 1987). Das Wachstum der Fronds verläuft unter optimalen Bedingungen exponentiell und die Verdopplungszeit beträgt ca. 2 Tage. Die Kultivation der Wasserlinsen im Labor kann entweder in flüssigen Medien oder auf verfestigten Substraten in Erlenmeyerkolben, Petrischalen oder Reagenzgläsern durchgeführt werden (Augusten und Gebhard 1988).

Die geringe Größe zusammen mit der vegetativen Vermehrung bei relativ hoher Vermehrungsrate sind die Gründe, weshalb Wasserlinsen einfach im Labor kultivierbar sind und verstärkt zur Bearbeitung physiologischer, biochemischer und ökologischer Fragestellungen eingesetzt werden (Wang 1986, 1990).

Ziel dieser Arbeit war es, folgende Fragen zu klären:

- Läßt sich die Notwendigkeit eines zusätzlichen Biotestes mit einer höheren Wasserpflanze für Fragen der Chemikalienbewertung und Abwasserprüfung demonstrieren?
- Eignet sich *Lemna minor* für die Etablierung eines standardisierbaren und hinreichend sensitiven Biotestverfahrens?

Hierfür wurden einerseits verschiedene Kultivationsbedingungen und Expositionsregime erprobt und andererseits Untersuchungen mit herbiziden Wirkstoffen und Umweltproben im Vergleich zu einem Algentest durchgeführt.

2 Material und Methoden

2.1 Kultivations- und Testbedingungen

Im Rahmen der vorliegenden Arbeit wurden Versuche mit der kleinen Wasserlinse *Lemna minor* L., bezogen von der Stammhaltung der Universität Jena (Prof. Jungnickel), durchgeführt. Die Lemnakultur wurde photoautotroph in sterilisiertem Nährmedium nach Steinberg (Tabelle 2-1) (Steinberg, 1946) kultiviert, das hinsichtlich der Verwendung von Natrium- anstelle von Ammoniummolybdat modifiziert wurde. Zur Anzucht wurden die Fronds von Schrägagarkulturen in Flüssigmedium angeimpft und für 14 Tage im Licht-Dunkel-Wechsel 14:10 Stunden angezogen, bevor sie für die Versuche verwendet wurden.

Die Kultivierung erfolgte in Präparatgefäßen mit geschliffenem Deckel, die sich in einem Wasserbad befanden. Die Temperatur des Wassers wurde mit Hilfe eines Thermostates bei 23 °C ± 2 °C konstant gehalten. Die Belichtung erfolgte von der Seite durch 4 Leuchtstoffröhren (je zwei Osram L36W/41 Interna und 2 Osram L36W 11 Daylight, Osram, Berlin), die auf der Oberfläche der Kulturgefäßen eine Lichtintensität von 35 W/m^2 gewährleisteten.

Tab. 2-1.: Zusammensetzung des modifizierten Steinberg-Mediums

Stoff		**Nährmedium nach Steinberg**	
Makroelemente	Molgew.	mg/l	mmol/l
KNO_3	101,12	350,00	3,46
$Ca(NO_3)_2*4H_2O$	236,15	295,00	1,25
KH_2PO_4	136,09	100,00	0,74
$MgSO_4*7H_2O$	246,37	100,00	0,41
Mikroelemente	Molgew.	mg/l	mmol/l
H_3BO_3	61,83	120,00	1,94
$ZnSO_4*7H_2O$	287,43	180,00	0,63
$Na_2MoO_4*2H_2O$	241,92	44,00	0,18
$MnCl_2*4H_2O$	197,84	180,00	0,91
$FeCl_3*6H_2O$	270,21	760,00	2,81

EDTA (Titriplex III)	372,24	1500,00	4,030

Die Biotests wurden über eine Zeitdauer von 7 Tagen durchgeführt. Die Temperatur betrug 23 °C ± 1 °C und wurde mit Wasserbadthermostatisierung konstant gehalten. Die Lichtintensität betrug 12 W/m^2 und erfolgt von oben unter Dauerlicht.

Es wurden spezielle Bechergläser ohne Tülle mit Glasdeckel und einem Nennvolumen von 100 ml eingesetzt. In jedes Glas wurden 60 ml Flüssigkeit gegeben. Bei jedem Test wurden Doppelproben mit definierten Stoffkonzentrationen, 4 Kontrollen und 2 sogenannte "Positivkontrollen" mit $K_2Cr_2O_7$ mitgeführt.

Die Verdünnungsreihen für die Schadstoffkonzentrationen wurden so berechnet, daß bei den Testorganismen möglichst eine 0 bis 100 %ige Hemmung der Wachstumsrate erzielt wurde.

Die Herstellung der Testansätze richtete sich nach dem folgenden Schema::

Anzucht der Lemnakultur ca.14 Tage vor Versuchsbeginn

⇓

Sterilisieren von 800 ml 2fach konzentriertem Steinberg-Nährmedium

⇓

Zugabe von jeweils 30 ml Schadstofflösung bzw. bidest.-Wasser

⇓

Überführen von 7 Fronds gleicher Größe in jedes Testgefäß

⇓

Einstellen aller Testgefäße in die Testanlage

⇓

Frondzahlermittlung alle 2 Tage

⇓

Bestimmung des Chlorophyllgehaltes

nach 7 Tagen Testdauer

⇓

Testauswertung – Ermittlung der Konzentrations-Wirkungs-Funktion

2.2 Testparameter

Alle für die Auswertung der Tests vorgesehenen Parameter wurden mit Hilfe von drei Meßgrößen bestimmt, nämlich:

- Frondzahl;

- Chlorophyllgehalt;
- Frischgewicht.

Die Frondzahl wurde alle 48 Stunden bestimmt, die zwei weiteren Parameter nach dem Ende des Tests - nach 7 Tagen.

Die Zählung der Wasserlinsenfronds erfolgte manuell. Aus der Bestimmung der Frondzahl lassen sich die folgenden Testparameter berechnen:

- Frondzuwachs;
- Vermehrungsrate;
- Wachstumsrate.

Der Frondzuwachs (FZ) errechnete sich wie folgt:

$$FZ = (F_n - F_0) / F_0 ,$$

die Berechnung der Wachstumsrate (μ) erfolgte nach der Gleichung :

$$\mu = (\ln F_n - \ln F_0) / t_n$$

wobei,

F_0 = Frondzahl am Anfang des Tests;

F_n = Frondzahl am Testende; und

t_n = Testdauer in Tagen.

Der toxische Einfluß von getesteten Chemikalien auf *Lemna minor* wurde als Hemmung der Wachstmsrate bzw. des Frondzuwachses bestimmt mit:

$$H = \mu_K - \mu_T / \mu_K) * 100 , [\%]$$

wobei,

H = Hemmung der Wachstumsrate/Frondzuwachs im Vergleich zur Kontrolle;

μ_K = Wachstumsrate bzw. Frondzuwachs der Kontrolle;

μ_T = Wachstumsrate bzw. Frondzuwachs der Testansätze.

Die so ermittelten Hemmwerte wurden bei der Herstellung von Konzentrations-Wirkungs-Beziehungen verwendet. Zur Erstellung der Konzentrations-Wirkungs-Kurven wurde eine HILL-Funktion der Form

$$E = Min + (Max - Min) / (1 + ((X / X50)^{-p}))$$

benutzt, wobei:

E = Effekt;

Min, Max = minimaler bzw. maximaler Effekt;

X = Konzentration;

X50 = mittlerer Effekt (Max-Min)/2; und

P = Steigung.

2.3 Bestimmung des Chlorophyllgehaltes

Als zusätzlicher Parameter bei der Auswertung der Biotests wurde der Chlorophyllgehalt bestimmt. Am Ende des Versuches, nach der Frondzahlbestimmung, wurden zuerst alle Fronds von allen Testansätzen mit saugfähigem Papier abgetrocknet und gewogen, damit ein Frischgewicht (FG) jeder Probe erhalten wurde. Die Chlorophyllbestimmung (gesamt Chlorophyll a und b) erfolgte photometrisch nach mechanischem Aufbruch der Fronds durch mörsern und einstündige Extraktion der Pigmente mit Aceton bei Raumtemperatur (nach Lichtenthaler und Wellburn 1983). Die Extinktion wurde mit Hilfe von einem Spectralphotometer (Perkin Elmer UV/VIS, Lambda 2S, Landau, FRG) bei 645 und 662 nm gemessen. Unter Verwendung der spezifischen Absorptionskoeffizienten für Chlorophyll a+b konnte die Gesamtchlorophyllmenge in µg/ml Acetonextrakt wie folgt errechnet werden:

$$C_a = (11{,}75\ E_{662} - 2{,}35\ E_{645}) / 5 \quad , [\mu g/ml]$$

$$C_b = (18{,}61\ E_{645} - 3{,}96\ E_{662}) / 5 \quad , [\mu g/ml]$$

Aus dem Chlorophyllgehalt im volumendefinierten Acetonextrakt wurde der Gehalt an Chlorophyll a+b pro mg Frischgewicht der Lemnapflanzen berechnet. Die Daten wurden für die Berechnung der Chlorophyllbiosynthesehemmung analog zur Berechnung der Wachstumshemmung ausgewertet.

2.4 Chemikalien

Informationen über die Herkunft und Reinheit der Testchemikalien sind in der Tabelle 2-2 zusammengestellt.

Tab. 2.-2: verwendete Testchemikalien

Name	CAS RN	Reinheit	Bezugsquelle
2,4-D	94-75-7	99 %	Riedel-de-Haen
Dichlobenil	1194-65-6	99 %	Riedel-de Haen
Kaliumdichromat	7778-50-9	Titrisol^R	Merck
Paraquat	1910-42-5	99 %	Riedel-de Haen

Zur Erprobung der Testbarkeit von komplexen Testgütern wurden Wasserproben aus dem Flußwasser der Neiße im Lemnatest eingesetzt. Es wurden 10 Proben von verschiedenen Standorten getestet, die linksseitig von der Quelle bis unterhalb Görlitz genommen wurden. Zu den Proben wurde ein 2fach konzentriertes Steinberg-Nährmedium zugegeben, so daß die Proben zur Hälfte verdünnt waren. Genauso wie bei der Einzelstofftestung wurden zusätzlich 4 Kontrollen und 2 Positivkontrollen im Test eingesetzt.

3 Ergebnisse

3.1 Testbedingungen

Zunächst werden die Untersuchungen zu den Kultivationsbedingungen für einen Lemnabiotest dargestellt.

Lemna minor wurde in zwei verschiedenen Nährmedien - in Grimme-Bordmann und in Steinberg-Medium - auf das Populationswachstum hin untersucht. Das Grimme-Bordmann-Medium (Grimme und Boardmann 1972) gilt als physiologisches Kultivationsmedium für Algen, während das Steinberg-Medium speziell für die Lemna-Kultivation empfohlen (Pluta und Maiwald 1996) wird.

Schon nach dreitägiger Kultivierung wurde festgestellt, daß die Vermehrung der Pflanzen im Grimme-Bordmann-Medium retardiert war, die Fronds ausbleichten und vergleichsweise klein blieben. Im Steinberg-Medium (Abbildung 3-1) verlief das Wachstum der Pflanzen bis zu 10 Tagen ohne Auffälligkeiten exponentiell. Nach einer längeren Kultivierung von ca. drei Wochen ergaben sich allerdings Probleme mit dem Wachstum von Algen in der Lemna-Kultur. Die Verdopplungszeit betrug in der exponentiellen Phase 2,2 d, was in etwa einer Verzehnfachung der Frondzahl in 7 Tagen bedeutet und den Ansprüchen der vorgeschlagenen OECD-Richtlinie (1997) genügen würde.

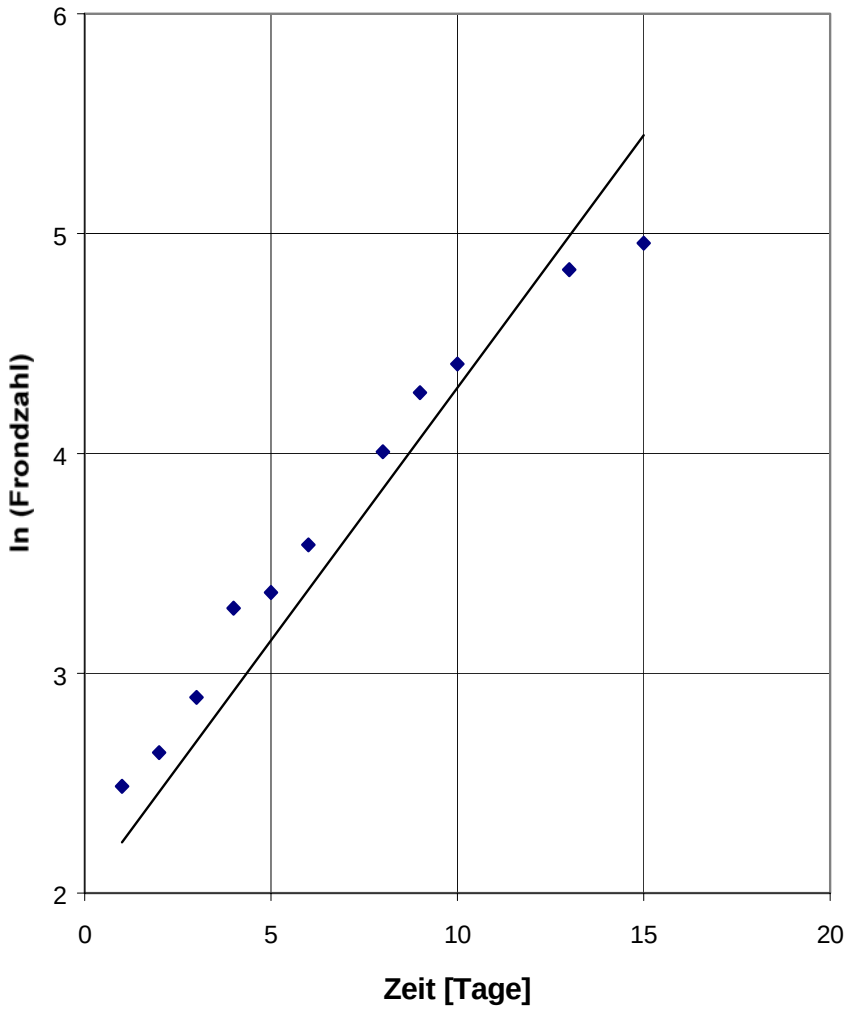


Abb. 3-1: Frondzahlentwicklung von *Lemna minor* in Steinberg-Medium, Kultivationsbedingungen wie in Material und Methoden beschrieben.

Im zweiten Schritt wurde das Pflanzenwachstum im Steinberg-Medium bei verschiedenen pH-Werten zwischen pH 4,5 und pH 10 untersucht. Da der pH-Wert des Steinberg-Medium 4,0 beträgt, wurden die Anfangs-pH-Werte der zu untersuchenden Proben durch

Zugabe von 1 N NaOH eingestellt. Nach 7 Tagen wurde eine geringfügige Erhöhung der pH-Werte beobachtet. Die Differenzierung des pH-Wertes des Mediums hatte keinen deutlichen Einfluß auf das Wachstum der Pflanzen.

Um die Reproduzierbarkeit sowie Ansprechempfindlichkeit der Biotestergebnisse einschätzen zu können, wurden zwei Kontrollkarten angelegt. Einerseits wurde die Wachstumsrate der unbehandelten Kontrollen verfolgt. Sie lag nach zehn unabhängigen Versuchen bei 0.224 ± 0.036 (Abb 3-2). Andererseits wurde Kaliumdichromat als Referenzchemikalie bei der Testung mit *Lemna minor* entsprechend den voräufigen Empfehlungen der OECD (1997) erprobt.

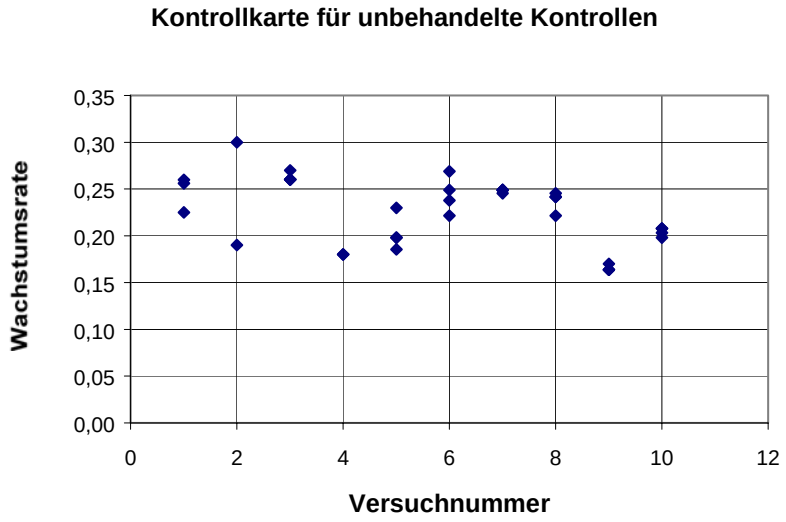


Abb. 3-2: Wachstumsrate der unbehandelten Kontrollen in zehn unabhängigen Versuchen.

Bei Kaliumdichromat handelt es sich um ein unspezifisch wirkendes Agens, das eine Wachstumshemmung zur Folge hat, wenn ein bestimmter Schwellenwert überschritten wird. Es wurde eine Verdünnungsreihe von 360 mg/l bis 0,02 mg/l $K_2Cr_2O_7$ hergestellt und die Konzentrations-Wirkungs-Beziehungen für die verschiedenen Parameter ermittelt. Abbildung 3-3 stellt die Daten und Funktion der für den Frondzahlzuwachs ermittelten Konzentrations-Wirkungs-Beziehung dar.

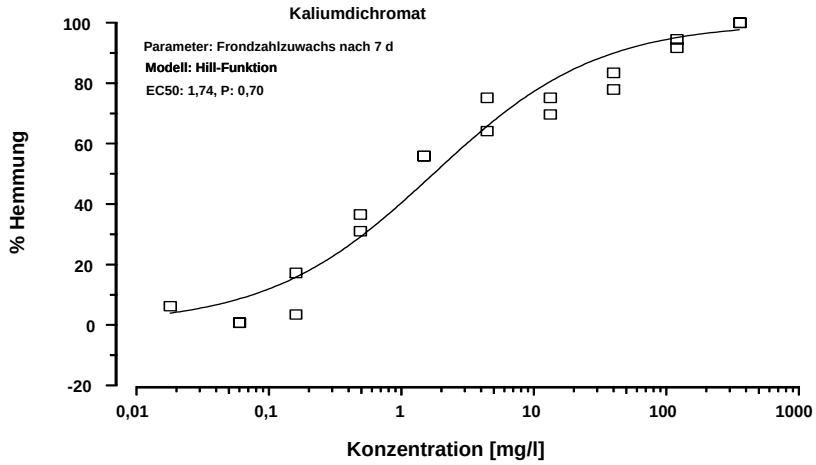


Abb. 3-3: Hemmung des Frondzuwachses von *Lemna minor* nach 7 Tagen durch Kaliumdichromat

Für die weiteren Tests wurde der EC_{50}-Wert (1,8 mg/l) der Frondzuwachshemmung als Positivkontrolle verwendet. Auf der Basis der geschätzten EC50 von 1.8 mg/l wurde eine Kontrollkarte erstellt, welche die Ansprechempfindlichkeit der Einzelteste kontrollieren soll. Die Ergebnise aus sechs unabhängigen Versuchen sind in Abbildung 3-4 wiedergegeben.

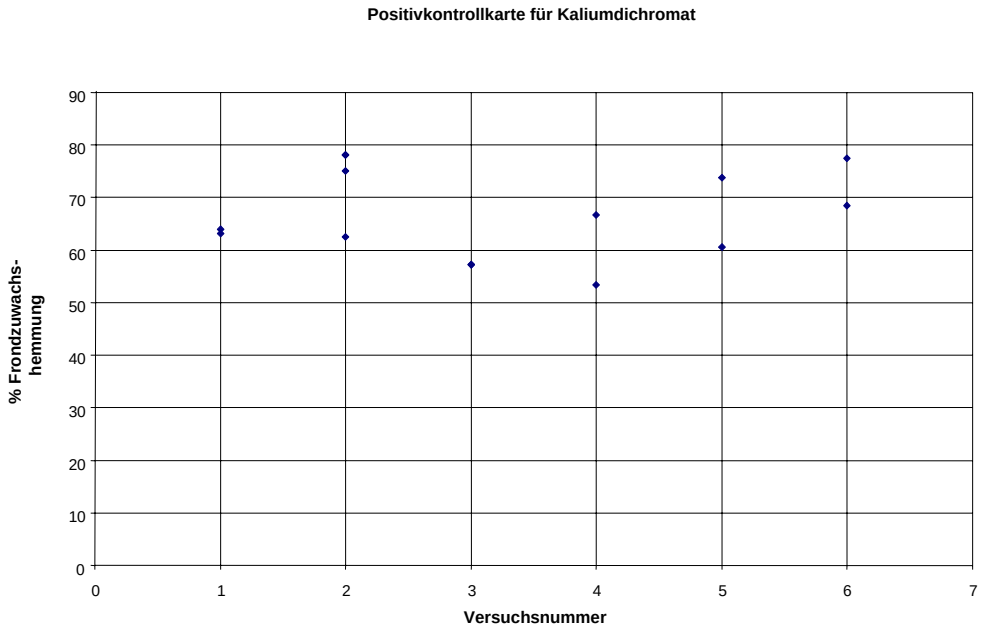


Abb. 3-4: Positivkontrollkarte für die Frondzuwachshemmung bei *Lemna minor* nach 7 Tagen Exposition mit 1.8 mg/l Kaliumdichromat.

Neben dem wachstumshemmenden wurden auch Effekte von Kaliumdichromat auf den photosyntheserelevanten Pigmentgehalt beobachtet. Die Fronds waren schon nach 2 Tagen Testdauer ausgebleicht und bei der Chlorophyllgehaltbestimmung wurde mit steigender $K_2Cr_2O_7$ - Konzentration immer weniger Chlorophyll erhalten.

3.2 Chemikalientestung

Nach Etablierung der Kultivations- und Biotestbedingungen wurden drei ausgewählte herbizide Wirkstoffe, nämlich 2,4-D, Dichlobenil und Paraquat, einer vergleichende Sensitivitätsuntersuchung mit einem Eingenerationentest mit *Scenedesmus vacuolatus* nach Altenburger et al. (1990) unterzogen.

2,4-D (2,4-Dichlorphenoxyessigsäure) repräsentiert einen Wirkstoff aus der Gruppe von Herbiziden mit Auxin-Wirkung (phytohormonelle Wirkung). Sie wurde in Deutschland als selektives Herbizid zur Bekämpfung dikotyler Unkräuter in Getreide eingeführt (Hock et al. 1995). Deutlich zu sehen ist eine erhöhte Empfindlichkeit des Lemna-Biotestes im Bereich niedriger Effektkonzentrationen, die auf höheren Effektniveaus allerdings konvergiert. Ursache hierfür ist eine vergleichsweise steilere Konzentrations-Wirkungs-Funktion im Algentest. Auf dem EC_{50}-Effektniveau ist immerhin noch eine Größenordnung Empfindlichkeitsunterschied - 16 im Vergleich zu 270 mg/l - zu

konstatieren. Für den Parameter Pigmentgehalt ist bei niedrigeren Konzentrationen an 2,4-D eine Förderung zu sehen. Bei der höchsten Konzentration wurde die Chlorophyllsynthese schwach gehemmt.

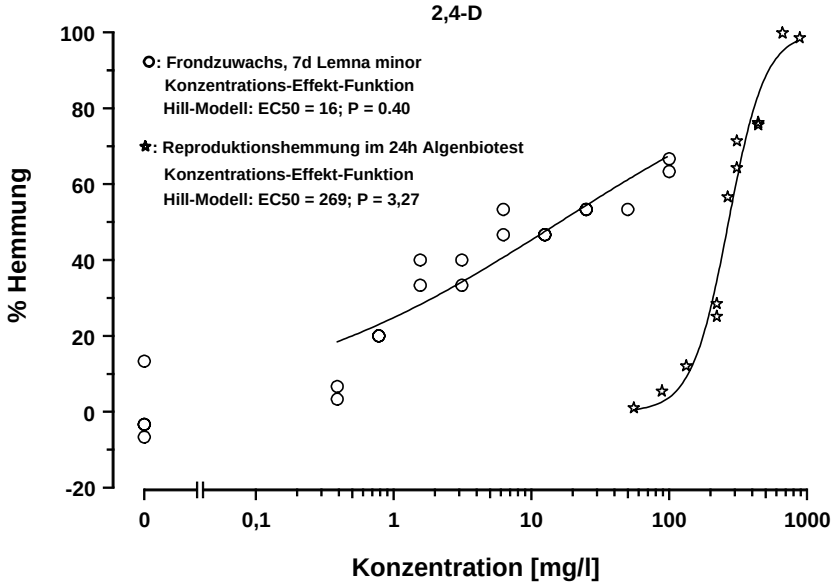


Abb. 3-5: Konzentrations-Wirkungs-Daten und Funktionen für 2,4-D im Algenreproduktionstest nach Altenburger et al. (1990) und im 7-Tage-Lemna-Biotest für den Parameter Frondzuwachs.

Dichlobenil ist ein Herbizid, dessen Wirkung auf einer Hemmung der Cellulose-Synthese beruht. Er wird zur totalen Unkrautbekämpfung in Nichtkulturland und teilweise im Weinbau angewendet (Hock et al. 1995). Abbildung 3-6 zeigt die Biotestergebnisse für *Scenedesmus vacuolatus* und *Lemna minor*. In Konzentrationen bis 10 mg/l zeigt dieses Herbizid keine Effekte auf die Reproduktion der Algen während die Wasserlinsen eine konzentrationsabhängige Hemmung des Wachstumsrate schon ab 30 µg/l aufweisen bei einem EC_{50} von 66 µg/l.

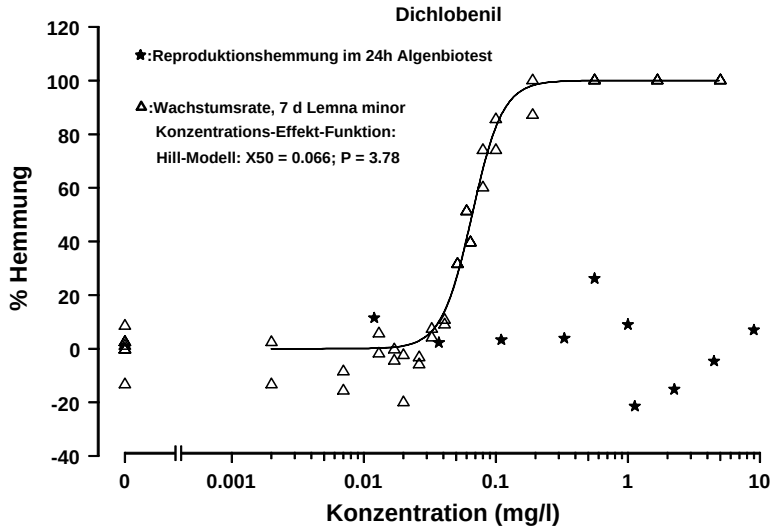


Abb. 3-6: Konzentrations-Wirkungs-Daten und Funktionen für Dichlobenil im Algenreproduktionstest nach Altenburger et al. (1990) und im 7-Tage-Lemna-Biotest für den Parameter Wachstumsrate.

Die Ergebnisse zeigen darüberhinaus eine Tendenz der Chlorophyllsynthesehemmung. Im Gegensatz zum Frondwachstum war der Chlorophyllgehalt allerdings nur bei höheren Konzentrationen beeinflußt.

Paraquat gehört zu der Gruppe der Redox-Verbindungen (Bipyridine), die vom Photosystem I (PS I) Elektronen übernehmen und dadurch zu Photooxidationen im Pflanzengewebe führen (schnelle herbizide Wirkung). Er tötet bei Sproß- und Blattapplikation alle grünen Pflanzen ab. Deswegen liegt sein Anwendungsbereich vornehmlich bei der totalen Vegetationskontrolle im Obst- und Weinbau und in Plantagenkulturen (Hock et al. 1995). Abbildung 3-7 stellt die Resultate der vergleichenden Untersuchungen dar. Wiederum weist der höhere Pflanzenbiotest mit *Lemna minor* eine deutlich höhere Empfindlichkeit im Vergleich zum Algentest aus. Für diesen Fall scheiden im Gegensatz zur Testung mit 2,4-D sowohl die bekannte Wirkungsspezifität als auch die pH-Abhängigkeit als einfache Erklärungen für den beobachteten Sensitivitätsunterschied von fast einer Größenordnung aus.

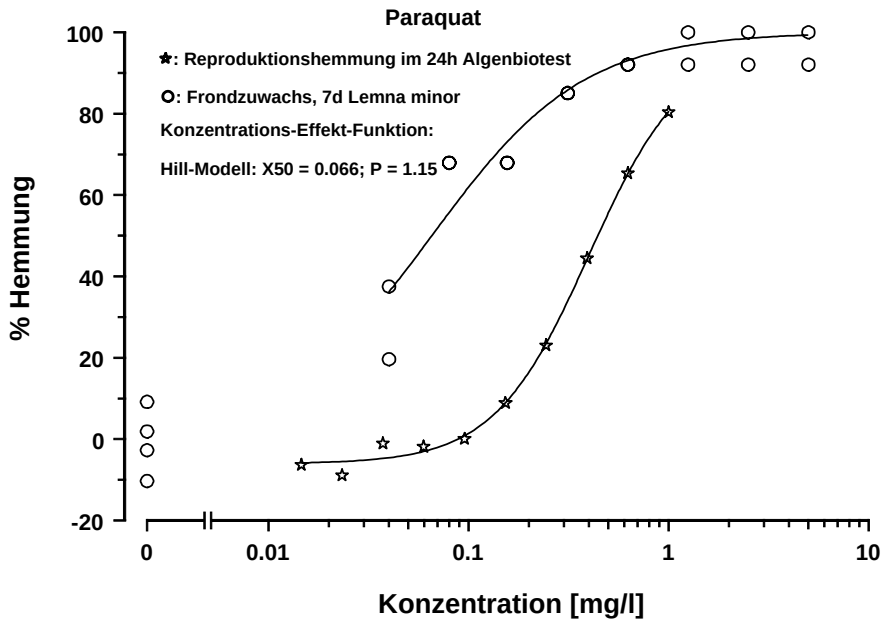


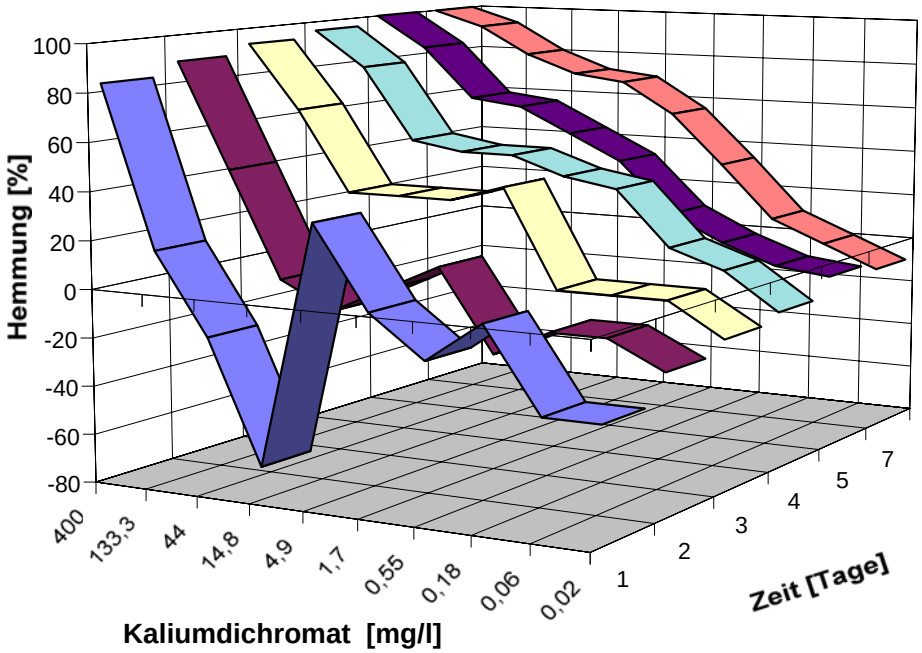
Abb. 3-7: Konzentrations-Wirkungs-Daten und Funktionen für Paraquat im Algenreproduktionstest nach Atenburger et al. (1990) und im 7-Tage-Lemna-Biotest für den Parameter Wachstumsrate.

Im Gegensatz zu Dichlobenil ist bei Paraquat darüberhinaus eine chlorophyllbiosynthesehemmende Wirkung (nicht dargestellt) der getesteten Chemikalie zu sehen, die konzentrationsmäßig niedriger als die Frondwachstumshemmung liegt.

3.3 Zeitabhängigkeit der Ergebnisse

Für die Auswertung aller Bioteste und die Ermittlung der Kennwerte der Konzentrations-Wirkungs-Beziehungen wurde der Zeitpunkt von 7 Tagen gewählt. In der Literatur (Lewis

1995) werden als Beobachtungszeitpunkt für Lemnatests der Zeitraum zwischen 4 und 14 Tagen genannt. In der Abbildung 3-8 ist exemplarisch für Kaliumdichromat dargestellt, wie sich die Hemmung der Frondzuwächse in Abhängigkeit von der Zeit verändert. Schon



nach ca. 4 Tagen Testdauer scheinen die Hemmwerte stabil zu bleiben. Dieses Ergebnis gilt qualitativ auch für die getesteten Wirkstoffe.

Abb. 3-8: Zeitabhängigkeit der Effektausprägung für die Hemmung des Frondzuwachses bei *Lemna minor* unter Kaliumdichromatbelastung.

3.4 Testung von Umweltproben

Als letzter Schritt der Arbeit wurden Umweltproben untersucht. Hierfür wurden Wasserproben aus der Neiße getestet. Die Neiße ist ein Fluß, der im Grenzgebiet zwischen Deutschland, Polen und der Tschechischen Republik fließt. Es wurden 10 Proben von verschiedenen Standorten genommen, die ein Längsprofil vom Quellgebiet bis kurz vor der Mündung in die Oder (unterhalb Görlitz) ergaben und diese vergleichend im Algentest und Lemnatest getestet. Alle Umweltproben zeigten ausschließlich fördernde Effekte auf die Alge *Scenedesmus vacuolatus* im 24 h Reproduktionshemmtest. Die Effekte der Wasserproben auf das Wachstum von *Lemna minor* sind in Abbildung 3-9 dargestellt. Es wurde eine Hemmung der Wachstumsrate zwischen 10 und 50% ermittelt, mit einem Schwerpunkt der wachstumshemmenden Einflüsse in der Umgebung von Zittau.

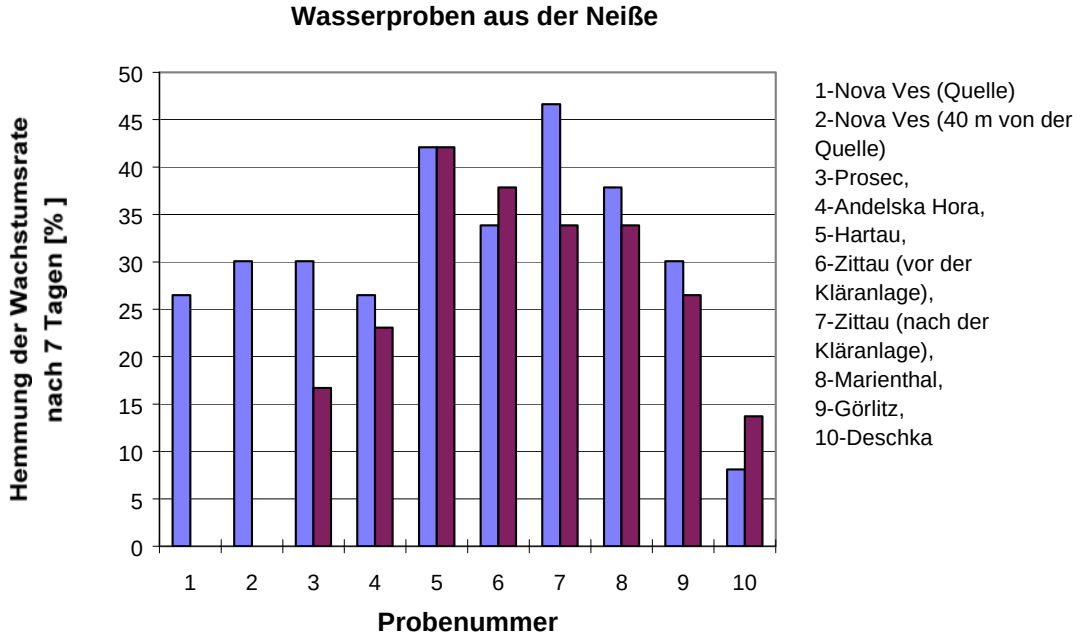


Abb. 3-9: Hemmung der Wachstumsrate von *Lemna minor* nach siebentägiger Exposition mit Wasserproben von verschiedenen Standorten entlang der Neiße im Vergleich zur unbehandelten Kontrolle.

4 Diskussion

Ziel dieser Arbeit war es, die Frage zu klären, ob die Wasserlinse *Lemna minor* ein geeigneter Testorganismus für die Prüfung von Umweltchemikalien und Umweltproben ist. Zur Beantwortung sollen an dieser Stelle die verschiedenen Milieubedingungen und Testparameter reflektiert, das Expositions- und Beobachtungsregime betrachtet sowie die Testergebnisse im Vergleich zu Algenbiotesten eingeordnet werden.

Die optimierten Testbedingungen liegen hinsichtlich der Zusammensetzung des Nährmediums, des pH-Regimes, der gewählten Beleuchtungsstärken und der Temperatur im Bereich der in der Literatur genannten Daten (ASTM 1991, Cowgill et al. 1991, Lewis 1995, OECD 1997). Von Bedeutung scheint auch das Alter der in den Tests eingesetzten Vorkultur. Definiert als Zeitraum beginnend mit der Überimpfung der Fronds von Agar-Nährlösung auf das flüssige Nährmedium bis zum Anfang des Tests, erhielten Christen und Theuer (1996) die größte Sensitivität der Lemnabiotests für 14 Tage alte Kulturen. Für die in dieser Arbeit durchgeführten Tests wurden daher in der Regel 14 Tage alte Kulturen verwendet.

Die Frondzahlbestimmung wird oft als ein Hauptkriterium bei der Auswertung von Lemnatesten gewählt (APHA, AWWA und WPCF 1989, Cowgill et al. 1991, Lewis 1995, Rodinger und Zach 1996, Hay 1996, Sallenave und Fomin 1997, Wang 1986). Hay (1996) bestätigt die Bestimmung der Frondzahl als ein leicht meßbares Wirkungskriterium im Vergleich zum Algentest. In ihrer Arbeit vergleicht sie u.a. drei pflanzliche Bioteste, nämlich einen Algen-, Wasserlinsen- und Kressetest. Der Wasserlinsentest hebt sich besonderes durch seine einfache Realisierbarkeit und gute Schadbilderkennung hervor. Die Wasserlinsenfronds sind hinreichend groß, um ihre Zahl auch in trüben Proben bestimmen zu können. Zusätzlich ist es möglich, die Änderungen der Fronds unter Einfluß des Schadstoffes festzustellen. Die Frondzahlbestimmung ist ein brauchbarer Parameter für die

Bewertung von Chemikalien oder Umweltproben. Von der Frondzahl kann der Frondzuwachs, die Wachstumsrate oder die Vermehrungsrate der Lemnakultur berechnet und eine quantitative Analyse durchgeführt werden. Diese Auswertungen zeigen allerdings keine qualitativen Änderungen der Pflanzen unter der Schadstoffwirkung an. Für derartige Zwecke werden Beobachtungen der Frondgröße, Wurzellänge, Frondfarbveränderungen oder Kolonieauflösungen eingesetzt. Oft werden neben der Frondzählung das Trockengewicht und der Chlorophyllgehalt gemessen oder die Blattfläche bestimmt. Cowgill et al. (1991) empfehlen eine Trockengewichtsbestimmung neben der Frondzählung. Taraldsen und Norberg-King (1990) führten als zweiten Parameter neben der Frondzählung eine Chlorophyllgehaltsbestimmung ein. Jenner und Jannsen-Mommen (1993) ermitteln über indirekte Battflächenbestimmung (Bildanalyse) eine höhere Sensitivität der Wasserlinsen gegenüber Schadstoffen. Auch Grossmann et al. (1992) benutzen diese Methode zur Auswertung von Wasserlinsentesten. Allerdings sind Meßgrößen wie das Trockengewicht und der Chlorophyllgehalt im Vergleich zur Frondzahlbestimmung nur destruktiv meßbare Wirkungskriterien und verlangen daher eine Endpunktfestlegung.

Hinsichtlich der Festlegung eines begründeten Expositions- und Beobachtungszeitraums sind die vorfindlichen Bioteste mit Wasserlinsen in der Regel schlecht begründet. In der Literatur finden Testzeiträume zwischen 4-14 Tagen Verwendung (Lewis 1995), wobei die Frondzahl zum Teil mehrfach, die anderen Parameter jedoch als Einpunktauswertungen ermittelt werden. An biologischen Begründungen für die gewählten Meßzeitpunkte fehlt es, hingegen wird nach Praktikabilitätsgesichtspunkten gehandelt. Dies gilt auch für diese Arbeit, die mit einem Expositions- und Beobachtungszeitraum von sieben Tagen im üblichen Rahmen liegt. Nur für auf Wachstumsraten bezogene Auswertungen ist ein willkürliches Beobachtungsregime zulässig, sofern die Linearität der Wachtumsfunktion auch für die behandelten Kulturen gezeigt werden kann. Dies wiederum erfordert allerdings mindestestens vier differenziert beurteilbare Meßzeitpunkte. In dieser Arbeit konnte zwar für die eingesetzten Stoffe gezeigt werden, daß die Zeit-Effekt-Kurven nach dem vierten Expositionstag für den Frondzuwachs stabil erschienen, was jedoch eine stoffspezifische Eigenschaft sein kann. Eine weitergehende Betrachtung dieser Frage könnte mit Hilfe des Modells des dynamischen Energiebudgets von Koijman und Bedaux (1996) erfolgen. Mit Hilfe dieses Modells sollten sich Stabilitätsbetrachtungen der Effektausprägung genauer und vor allem reproduzierbar gestalten lassen. Vorläufig scheint es daher sinnvoll, die Bestimmung der Frondzahl in 24 oder 48 h-Intervallen vorzunehmen, um eine Beurteilung der Wirkung von Schadstoffen in Abhängigkeit von der Zeit zu ermöglichen.

Die Kernfrage dieser Arbeit nach der Notwendigkeit eines zusätzlichen pflanzlichen Biotestes in der aquatischen Toxikologie wurde mit Hilfe des Einsatzes von spezifisch wirkenden Phytopharmaka bearbeitet. Die in Tabelle 4-1 zusammengestellten Daten zeigen, daß die Empfindlichkeit der Wasserlinsen im Vergleich zu Algen für die untersuchten Wirkstoffe nicht nur in dieser Arbeit, sondern auch nach Literaturangaben in der Regel höher ist. Die Einzelheiten können dabei kritischer Betrachtung hinsichtlich des Einflusses von Milieufaktoren wie dem pH-Wert bei der Testung einer Säure wie etwa 2,4-D oder dem Meßzeitraum unterzogen werden. Dies ändert allerdings nichts an der Stärke des pharmakologischen Arguments, das *Lemna* als mehrzelliger, höherer Organismus andere Angriffspunkte für Schadstoffe bietet als eine einzellige, niedere Alge, was insbesondere im untersuchten Fall der herbizidwirksamen Substanz Dichlobenil evident wird.

Tab. 4.1: Vergleich von Biotestdaten für Algen und Wasserlinsen für die untersuchten herbiziden Wirkstoffe

Stoff	Empfindlichkeit eigene Ergebnisse		Empfindlichkeit nach Grossmann et. al (1992)		Empfindlichkeit nach Fairchild et. al (1997)		Empfindlichkeit nach Fletcher (1990)	
	Algen	Wasser-linsen	Algen	Wasser-linsen	Algen	Wasser-linsen	Algen	Wasser-linsen
2,4-D	+	++	+	++	++	+	+	++
Dichlobenil	-	++	+	++				
Paraquat	+	++	+	++	+	++	+	+

- kein Effekt der Chemikalie im wasserlöslichen Bereich

\+ Effekt demonstriert

++ reagiert empfindlicher

Es gibt auch Chemikalien, bei denen eine größere Empfindlichkeit der Algen festzustellen ist. Fletcher (1990) stellte Daten der PHYTOTOX-Datenbank (Sensitivität von Algen und höheren Pflanzen gegenüber organischen Chemikalien) zusammen, und die Ergebnisse zeigen, daß es nicht möglich ist, eine von beiden Organismentypen generell als sensitiver zu bezeichnen.

Neben den pharmakologischen und botanisch-systematischen Rationalen zählen für die Frage nach der Notwendigkeit eines neuen pflanzlichen Biotestes aber auch praktische Gesichtspunkte. Laut Sortkjaer (1984) und Lewis (1995) ist die Testung von Umweltproben mit Hilfe von Wasserlinsen zu Zwecken der Früherkennung wie auch im Langzeitmonitoring von potentiellen Umweltschädigungen gut geeignet. Im Gegensatz zu Wasserlinsen zeigen Algen eine starke pH-Wert-Abhängigkeit im Wachstumsverhalten der unbehandelten Kontrollen (Peterson 1991) und damit in der Beurteilungsreferenz für stoffspezifische Effekte. Zusätzlich ergeben sich Schwierigkeiten bei der Einschätzung von Stoffen, wenn die Proben trüb oder gefärbt sind. Diese Faktoren haben ebenfalls einen hemmenden Einfluß auf das Wachstum der Algen, ohne toxisch zu sein, da der Lichtzutritt in die Probe und damit die Photosynthese erschwert wird (Wang 1990). Eine größere Empfindlichkeit der Wasserlinsen im Vergleich zu Algen bei der Umweltprobentestung beschreiben auch Sallenave und Fomin (1997). Sowohl bei Algen als auch bei Wasserlinsen ist zu berücksichtigen, daß einige Stoffe, die sich in den Umweltproben befinden, eine Förderung des Pflanzenwachstums bewirken können, was die Hemmung durch Schadstoffe überlagern kann. Insbesondere organische Nährstoffe vermögen diese Effekte hervorzurufen, da beide Organismengruppen in der Lage sind, sich mixotroph zu ernähren. Hier ist wiederum die Wahl physiologisch optimierter Wachstumsbedingungen angezeigt, um die resultierenden Probleme zu minimieren.

Mit Ausnahme der Chlorophyllbestimmung ist die Parameterbestimmung im Lemnabiotests im Vergleich mit anderen Biotests sehr einfach und nicht destruktiv. Die gute Auswertbarkeit des Lemnabiotests (Jenner und Janssen-Mommen 1993) gilt als ein großer Vorteil gegenüber anderen Biotesten, besonders in der Umweltprobentestung mit Algen, wo sich bei trüben Lösungen Probleme mit der Algenzellzahl- oder Fluoreszenzbestimmung ergeben und wo es nötig sein kann, die Proben zusätzlich vorzubereiten (z.B. durch Filtration) oder aufwendige Korrektureinrichtungen vorzuhalten.

Zusammenfassend sei empfohlen, nicht einen Testorganismus durch einen anderen zu ersetzen, sondern einen Wasserlinsenbiotest ergänzend in die aquatische Testbatterie aufzunehmen, um ein breiteres Spektrum von verschiedenartigen Chemikalienwirkungen erfassen zu können.

5 Zusammenfassung

In der vorliegenden Arbeit wird die Etablierung eines Biotests mit der kleinen Wasserlinse *Lemna minor* und die Biotestung von Einzelstoffen und Umweltproben mit Hilfe dieser Pflanze demonstriert.

Ein optimiertes Testsystem mit exponentiellem Wachstum der Lemnakultur wird für das Steinberg-Medium bei einer Kultivationstemperatur von 23 °C erreicht, wobei der Anfangs-pH-Wert in einem weiten Bereich keinen deutlichen Einfluß auf das Wachstum der Pflanzen hat. Als Beobachtungsparameter bewährt sich die zeitgestaffelte Zählung der Fronds. Als Expositions- und Beobachtungszeitraum wurden 7 Tage etabliert. Die Reproduzierbarkeit der Testergebnisse konnte für die Wachstumsrate der unbehandelten Kontrollen und für die Reaktion auf eine Positivkontrolle mit Kaliumdichromat gezeigt werden.

In der Biotestung von ausgewählten Einzelstoffen (Testung der herbiziden Wirkstoffe 2,4-D, Dichlobenil und Paraquat) konnte eine im Vergleich zu einem Algenbiotest höhere Empfindlichkeit nachgewiesen werden. Aus den Konzentrations-Wirkungs-Beziehungen für die drei Herbizide konnten die EC_{50}-Werte für Dichlobenil und Paraquat mit 0,066 mg/l und für 2,4-D mit 16 mg/l ermittelt werden.

Weiterhin wurden komplex belastete Wasserproben aus der Neiße mit Hilfe des Lemna- und Algentests untersucht. Im Gegensatz zum Algenbiotest wurde eine hemmende Wirkung durch die Umweltproben auf Wasserlinsen festgestellt. Die Hemmung betrug in einigen der 10 Probennahmeorten bis zu 50%.

In der Diskussion, ob die Ergänzung der Biotestbatterie im aquatischen Bereich um eine höhere Pflanze notwendig ist, werden folgende Vorteile der Wasserlinsen gegenüber Algen hervorgehoben:

- Lemna vermag als höhere Pflanze pharmakologisch andere Effekte als die niedrigen, einzelligen Algen zu erfassen;
- die Größe der Wasserlinsenfronds ermöglicht eine komplexe Schadbilderkennung (Bonitur) bei technisch unaufwendiger Meßbarkeit;
- Lemnaceen können in Nährlösungen unterschiedlicher pH-Stufen inkubiert werden - Algenbioteste müssen demgegenüber in einem engeren pH-Bereich verlaufen;
- Wasserlinsentests können in trüben oder gefärbten Testlösungen durchgeführt werden. Bei Algenbiotesten müssen die Proben vorbereitet werden, damit die Pflanzen genug Licht bekommen.

6 Literaturverzeichnis

Altenburger, R., Bödeker, W., Faust, M., Grimme, L.H. (1990): Evaluation of the isobologram method for the assessment of mixtures of chemicals. Combination effect studies with pesticides in algal biotests. Ecotoxicol. Environ. Safety 20: 98-114

APHA (American Public Health Association), AWWA (American Water Works Association), WPCF (Water Pollution Control Federation) (1989): Standard methods for the examination of water and wastewater. 18th edt. Sect.8220. Washington, DC

ASTM (American Society for Testing and Materials). (1991): Standard guide for conducting static toxicity test with *Lemna gibba* G3. American Society for Testing and Materials E 1415-91

Augusten, H. (1984): Lemnaceen, Aspekte ihrer Praxisrelevanz. Biol. Rdsch. 22: 225-234

Augusten, H., Gebhardt, A. (1988): Der Einfluß von Schwermetallen auf die Turionenbildung bei *Spirodella polyrhiza* L. Schleiden Wiss. Z. Päd. Hochsch. Potsdam 32: 29-33

Christen, O., Theuer, C. (1996): Sensitivity of *Lemna* bioassay interacts with stock-culture period. J. Chem. Ecol. 22: 1177-1186

Cowgill, U.M., Milazzo, D.P., Landenberger, B.D. (1991): The sensitivity of *Lemna gibba* G-3 and four clones of *Lemna minor* to eight common chemicals using a 7 day test. Res. J. Water Pollution Con. Fed. 63: 991-998

Fairchild, J.F., Ruessler, D.S., Haverland, P.S., Carlson, A.R. (1997): Comparative sensitivity of *Selenastrum capricornutum* and *Lemna minor* to sixteen herbicides. Arch. Environ. Contam. Toxicol. 32: 353-357

Fletcher, J.S. (1990): Use of algae versus vascular plants to test for chemical toxicity. ASTM STP 1091. in Wang W, Gorsuch, J W, Lower W R (Eds). American Society for Testing and Materials, Philadelphia. 33-39

Greenberg, B.M., Huang, X.-D., Dixon, D.G. (1992): Applications of the aquatic higher plant *Lemna gibba* for ecotoxicological assessment. J. Aquatic Ecosystem Health 1: 147-155.

Grimme, L.H., Boardman, N.K. (1972): Photochemical activities of a partical fraction P1 obtained from the green alga *Chlorella fusca*. Biochem. Biophys. Res. Commun. 49: 1617-1623

Grossman, K., Berghaus, R., Retzlaff, G. (1992): Heterotrophic plant cell suspension cultures for monitoring biological activity in agrochemical research. Comparisons with screens using algae, germinating seeds and whole plants. Pestic. Sci. 35: 283-289

Hay, A. (1996): Vergleichende Arbeiten an Biotesten und deren praktische Umsetzung am Beispiel von Eluaten aus Sonderabfällen. Diplomarbeit, Universität Hohenheim

Hock, B., Fedtke, C., Schmidt, R.R. (1995): Herbizide. Georg Thieme Verlag, Stuttgart, New York

Jenner, H.A., Jannsen-Mommen, J.P.M. (1993): Duckweed *Lemna minor* as tool for testing toxicity of coal residues and polluted sediments. Arch. Environ. Contam. Toxicol. 25: 3-11

Kanne, R. (1989): Biologische Toxizitätstest Teil II: Gegenwärtig zur Verfügung stehende Testverfahren. UWSF-Z. Umweltchem. Ökotox. 3: 23-26

Kooijman, S.A.L.M., Bedeaux, J.J.M. (1996) The analysis of aquatic toxicity data. Amsterdam, VU University Press, NL

Landolt, E., Kandeler, R. (1987). Biosystematic investigations in the family of duckweeds (*Lemnaceae*), Vol. 4. The family of *Lemnaceae* – a monographic study. Vol. 2. Veröff. Geobot. Institut der ETH Zürich, 95. Heft

Lewis, M.A. (1995): Use of freshwater plants for phytotoxicity testing: a review. Environ. Pollution 87: 319-336

Lichtenthaler, H.K., Wellburn, A.R. (1983): Determination of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Biochem. Soc. Trans. 591-592

Nusch, E.A. (1992): Grundsätzliche Vorbemerkungen zur Planung, Durchführung und Auswertung biologischer und ökotoxikologischer Testverfahren. In: Steinhäuser K G und P.-D. Hansen (Hrsg) Biologische Testverfahren. Stuttgart/New York: G Fischer S 35-48

OECD (Organisation of Economic Cooperation and Development) (1997): *Lemna* growth inhibition test. draft proposal

Peterson, H.G. (1991): Toxicity testing using s chemostat-grown green algae, *Selenastrum capricornutum.* Plants for toxicity assessment, ASTM STP 1115, American Society for Testing and Materials, Philadelphia, 107-117

Pluta, H.-J., Maiwald, D. (1996): persönliche Mitteilung zum Versuchprotokoll des Umweltbundesamtes Berlin

Rodinger, W., Zach, B. (1996): Wasserbeschaffenheit - Prüfung der Vermehrungshemmung von *Lemna minor* L. - Arbeitsanweisung des Österreichischen Bundesamtes für Wasserwirtschaft, Institut für Wassergüte

Ren, L., Huang, X.-D., McConkey, B.J., Dixon, D.G., Greenberg, B.M. (1994): Photoinduced toxicity of three polycyclic aromatic hydrocarbons (fluoranthene, pyrene and naphthalene) to the duckweed *Lemna gibba* L. G-3. Ecotoxicol. Environ. Saf. 28: 160-171

Sallenave, R., Fomin, A. (1997): Some advantages of the duckweed test to assess the toxicity of environmental samples. Acta Hydrochim. Hydrobiol. 25: 135-140

Sortkjaer, O. (1994): Macrophyte and Microphyte communities as test systems in ecotoxicological studies of aquatic systems. Ecological Bulletins 36: 75-80

Steinberg, R.A. (1946): Mineral requirements of *Lemna minor*. Plant Physiol. 21:42-48.

Taradelsen, J.E., Norberg-King, T.J. (1990): New method for determing effluent toxicity using duckweed (*Lemna minor*). Environ. Toxicol. Chem. 9: 761-767

Wang, W. (1986): Toxicity tests of aquatic pollutants by using common duckweed. Environ. Poll. Ser. B, 11: 1-14

Wang, W. (1990): Literature review on duckweed toxicity testing. Environ. Res. 52: 7-22

Kapitel IV

Exposition und Toxizität – Der Faktor Zeit in Routinebiotests

Rolf Altenburger und Thomas Backhaus
In: W. Mücke, M. Link (Hrsg.). *Biotests in der Praxis.* Institut für Toxikologie und Umwelthygiene, Technische Universität München. S. 61-74. (2000)

Exposition und Toxizität – Der Faktor Zeit in Routinebiotests

Rolf Altenburger[1], Thomas Backhaus[2]

[1]Sektion Chemische Ökotoxikologie
UFZ-Umweltforschungszentrum Leipzig-Halle GmbH
Permoserstraße 15, 04318 Leipzig

[2]Universität Bremen
Fachbereich Biologie/Chemie
Leobenerstraße, 28334 Bremen

In: W. Mücke, M. Link (Hrsg). *Biotests in der Praxis*, Institut für Toxikologie und Umwelthygiene, Technische Universität München. S. 61-74. (2000)

Abstract

Bioteste sind Experimente zur qualitativen oder quantitativen Erfassung von Lebensäußerungen von Organismen, die in Reaktion auf eine Exposition gegenüber einzelnen Stoffen oder Stoffgemischen erfolgen. Wenn die Zielsetzung des Einsatzes biologischer Testverfahren in der Praxis die Beurteilung von potentiell schädlichen Effekten ist, sollten die methodischen Randbedingungen hinsichtlich festgelegter Einwirkungs- und Auswirkungszeit vor einer Ergebnisbewertung reflektiert werden, um grobe Fehleinschätzungen der Toxizität zu vermeiden. An drei Fallsituationen werden mit verschiedenen Beispielen die Schwierigkeiten illustriert und erörtet die auftreten können, wenn es gilt einen quantitativen Zusammenhang zwischen Exposition und Toxizität herzustellen.

Für die Beurteilung der Ökotoxizität flüchtiger Verbindungen, wie z.B Chlorbenzole, sind viele der Routinebiotests nicht geeignet, da die eingesetzten Konzentrationen mit der Expositionszeit nicht reproduzierbar abnehmen. Technische Modifikationen oder kritische Sichtung von zeitabhängigem Verhalten bei Mehrfachbeobachtung können die Aussagequalität deutlich verbessern helfen.

Stoffe deren Wirkung auf z.B. der spezifischen Inhibierung von Biosynthesen, der Reproduktion und anderen bei Berücksichtigung der Lebensspanne biologisch erst langfristig bedeutsamen Prozessen beruht und Substanzen mit starken Ladungseigenschaften oder hoher Lipophilie sind in akuten Biotesten oftmals nicht sicher zu beurteilen. Viele Antibotika etwa zeigen ihre toxischen Effekte auf Leuchtbakterien erst bei längerer Auswirkungszeit.

Auch die Beurteilung von Stoffmischungen, wie sie etwa in der Abwassertestung üblich ist, muß mit zeitabhängigen Toxizitätsbefunden rechnen. Unkritisch ist dies für Gemische aus ähnlich wirkenden Komponenten, da hier ein Verhalten wie bei einzelnen Stoffen angenommen werden kann. Bei der Beurteilung von komplexen Belastungen mit Komponenten, die unterschiedliche Wirkweisen aufweisen, kann jedoch die tatsächliche Toxizität des Gemisches erheblich unterschätzt werden.

Einführung

Für der Beurteilung unerwünschter und schädlicher Effekte von Umweltchemikalien und Abwässern, Abgasen oder festen Abfällen ist der Einsatz biologischer Verfahren zur Charakterisierung und Quantifizierung von Schadpotentialen vielfach unverzichtbar. Von der Vielzahl etablierter biologischer Testverfahren sind einige als standardisierte Bioteste in der behördlich-regulativen Praxis der prospektiven und retrospektiven Stoffbeurteilung und Umweltüberwachung etabliert. Im Vergleich zu chemisch-analytischen Verfahren der

Quantifizierung des Auftretens von Chemikalien in der Umwelt, ist die biologische Beurteilung von Kontaminationen und Schadwirkungen jedoch noch immer relativ unterentwickelt.

Gerade die fortgesetzte ‚Überraschungsgeschichte' des Entdeckens von neuartigen, unerwünschten Chemikalieneffekten wie etwa hormonähnlichen Wirkungen durch chemische Fremdstoffe zeigt die Bedeutung rationaler biologischer Herangehensweisen für eine belastbare Beurteilung von ökotoxikologischen Wirkungspotentialen. Neben den qualitativen Anforderungen existieren jedoch auch quantitative Herausforderungen. So sind wie der Abbildung 1 zu entnehmen ist, selbst von Chemikalien die kommerziell in mehr als 1000 t/Jahr Verwendung finden (high production volume chemicals), bislang nur eine Minderheit im Hinblick auf basale Kriterien zur Bestimmung ihres Verhaltens in der Umwelt und ihrer Schadwirkungspotentiale für Organismen untersucht worden.

Diese Hinweise sollen zeigen, daß der rationalen Anwendung von Biotesten für die Zukunft noch vielfältige Aufgaben bevorstehen. Eine Dimension wird in der auf Bioteste gestützten Beurteilung der durch Fremdstoffe ausgelösten schädlichen Effekten oftmals vernachläßigt, nämlich der Faktor Zeit. Die Zeitdimension spielt sowohl als Einwirkzeit von Stoffen auf Organismen (Exposition) als auch als Auswirkzeit für die Entwicklung von beobachtbaren Effekten an Organismen eine hervorragende Rolle. Gerade in der Praxis der Routinebiotestung wird auf normierte Verfahren niedergelegt etwa als DIN-Vorschriften zurückgegriffen, in denen die Zeit als beeinflussende Beobachtungsgröße durch “Endpunktsfestlegung” ausgeblendet wird. Dieser Artikel will anhand von Beispielen an die Bedeutung des Faktors Zeit für die Beurteilung von ökotoxischen Schadwirkungen erinnern und Möglichkeiten einer routinetauglichen Berücksichtigung aufzeigen.

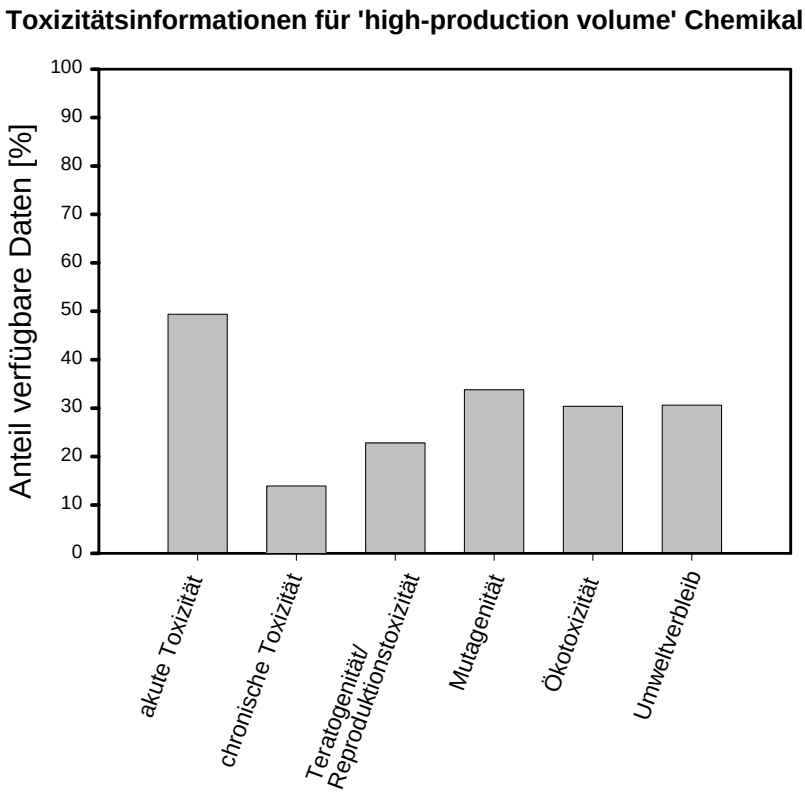


Abb.1: Noch immer liegen für die Mehrzahl selbst der mengenmäßig bedeutensten Substanzen nur unzureichende Informationen zu schädlichen Wirkpotentialen vor (nach Betts, 1998)

Biologische Testverfahren werden in diesem Beitrag verstanden als Methoden zur qualitativen oder quantitativen Erfassung von Lebensäußerungen, die in Reaktion auf eine Exposition gegenüber Stoffen erfolgen. In standardisierter Form etabliert, werden derartige Testverfahren auch als Biotests bezeichnet. Wirkung als zweiter wesentlicher Begriff, der in dieser Arbeit zugrundegelegt wird, bezeichnet die Summe aller Veränderungen in einem biologischen System, ausgelöst durch die definierte Menge eines Stoffes. Die Dimension der Zeit erfordert dabei die Berücksichtigung des Ausgangs- und Endzustandes des betrachteten biologischen Systems vor dem Hintergrund, daß alle biologischen Systeme sich in der Zeit entwickeln.

Einwirkungszeit

Die Feststellung, daß die Dauer die ein biologisches System einem Fremdstoff gegenüber ausgesetzt/exponiert ist und somit Gelegenheit zum Wechselwirken bietet von entscheidender Bedeutung für die Art und das Ausmaß beobachtbarer Effekte sein kann, scheint zunächst trivial. Physiko-chemische Prozesse vermögen die Einwirkungsdauer durch Zersetzungprozeße wie Hydrolyse, Thermolyse oder Photolyse und Verteilungsprozeße wie Sorption oder Verflüchtigung stark zu beeinflussen. Auch biotischer Stoffabbau oder Sorption können angenommene Stoffkonzentrationen verändern. Der chemisch-analytische Aufwand für eine Kontrolle der nominalen Stoffkonzentrationen über die Expositionszeiträume wird jedoch nur in seltenen Fällen betrieben. Bei Umweltproben mit unbekanten Kontaminationen oder multiplen Belastungen ist er gar oft völlig unmöglich. Im Ergebnis führen Probleme der einen oder anderen Art in der Regel zu nur schwer reproduzierbaren Testergebnissen und im schlimmsten Falle gar zu widersprüchlichen Toxizitätseinschätzungen.

Ist von einem Testgut zu erwarten, daß dessen Einwirkzeit auf den verwendeten Biotest nicht hinreichend zu definieren ist, bieten sich eine Reihe von Möglichkeiten durch technische Modifikationen am Biotest die Reproduzierbarkeit und Beurteilbarkeit der Ergebnisse zu verbessern:

- Flüchtige Substanzen sollten beispielsweise nach Möglichkeit in gasdichten oder kurzzeitigen Testsystemen untersucht werden;
- Proben mit hohen sorptiven Eigenschaften sollten nur mit Glasgefäßen und Glaspipetten (deren Oberflächen sich auch noch silanisieren lassen) bearbeitet werden;
- das Testgut kann vergleichend nach einer Standzeit, die der gewünschten Expositionszeit entspricht nochmals im Biotest eingesetzt werden, um über einen Wirksamkeitsvergleich zu einer Abschätzung der Expositionssituation zu gelangen.

Abbildung 2 zeigt eine weitere Möglichkeit wie mit Hilfe von zeitgestaffelter Mehrfachbeobachtung im Kurzzeit-Leuchtbakterientest die Einwirkzeit von zu untersuchenden Noxen beurteilt werden kann. Untersucht wurde die Leuchtbakterientoxizität von Monochlorbenzol, das in Bitterfelder Grundwasserproben immer wieder als Kontaminante auffällt. Die starke Neigung von Chlorbenzol zur Verflüchtigung läßt sich wiederfinden in einem mit der Meßzeit abnehmenden Effekt auf die Leuchtbakterienlumineszenz. Dieses Verhalten wird auch für viele der untersuchten Grundwasserproben aus dem Bitterfelder Raum beschrieben (Altenburger et al. 1999), was zeigt, daß ein willkürlich gesetzter Beobachtungszeitpunkt für den Biotest leicht zu Fehleinschätzungen der ökotoxischen Potenzen führen könnte.

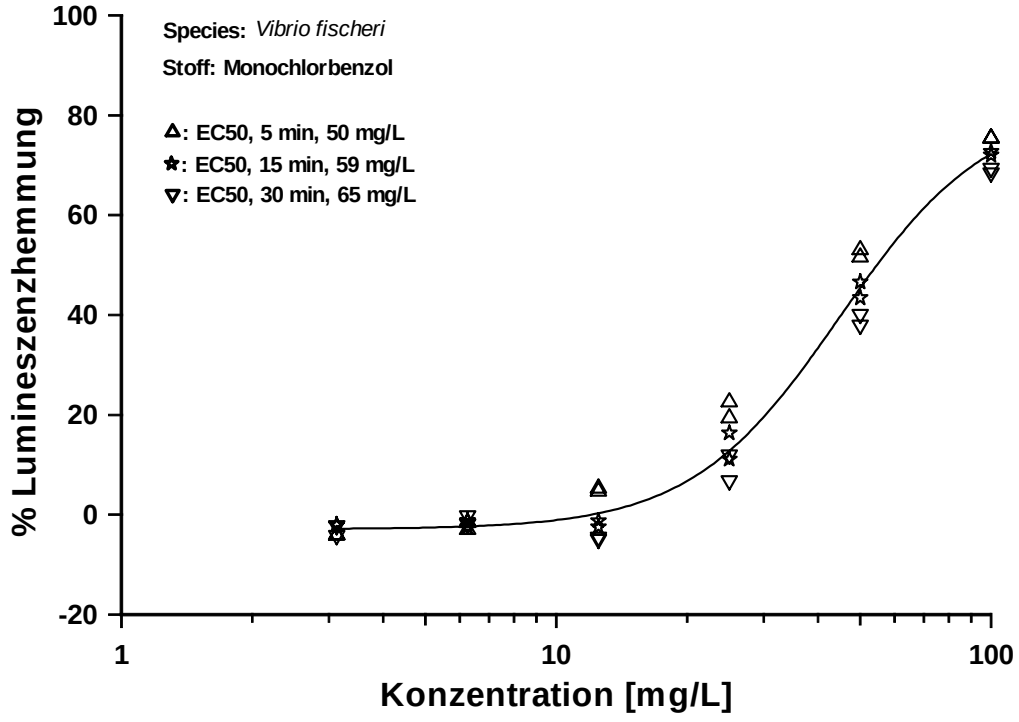


Abb.2 : Lumineszenzhemmung bei *Vibrio fischeri* durch Chlorbenzol in Abhängigkeit vom Meßzeitpunkt

Auswirkungszeit

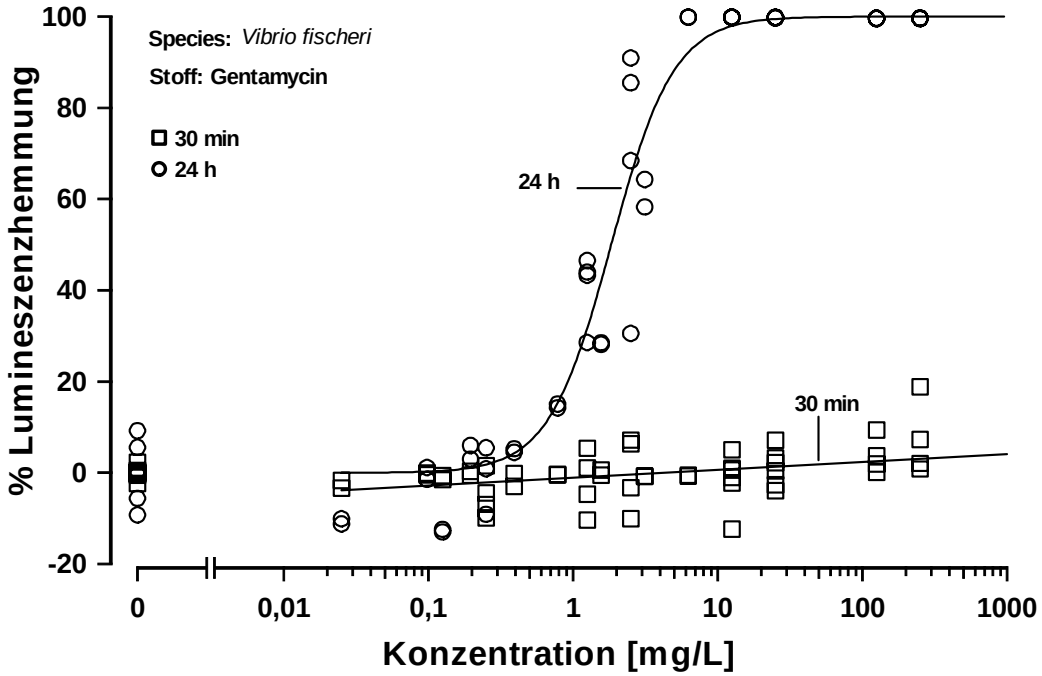
Die Zeit, die ein biologisches System benötigt bis eine Schadwirkung manifest und in Lebensäußerungen beobachtbar wird, kann in Abhängigkeit von der Art der Kontamination und der Qualität der Effekte stark variieren. Substanzen mit mittlerer Lipophilie, einer Größe bis zu einem Molekulargewicht von etwa 300 Dalton und direkten Effekten auf die strukturelle Integrität der Zelle, bzw. die energieerzeugenden Prozesse von Organismen lassen sich in der Regel schon nach kurzer Zeit, akut, erfassen und sicher quantifizieren. Als Probleme in der Beurteilung ökotoxischer Potenzen hinsichtlich der benötigen Zeit zum Erkennen der biologischen Auswirkungen eines Schadens hingegen sind bekannt:

- Substanzen die aufgrund starker Ladungseigenschaften oder hoher Lipophilie längere Zeit benötigen, um in exponierte Organismen zu gelangen bzw. eine Gleichgewichtskonzentration zu erreichen;
- Stoffe, die unspezifische subletale Effekte erzeugen;
- Chemikalien mit spezifischen Wirkungsmechanismen an Wirkorten, die die Homöostase nicht akut beeinträchtigen;
- Schadstoffgemische.

Für die beiden letztgenannten Fälle sollen im folgenden Beispiele ausgeführt werden. In den gegenwärtig geführten Debatte um das Auftreten von Arneimittelrückständen in Umweltmedien und möglichen ökotoxischen Effekten wird insbesondere auch die Gruppe der Antibiotika als bedeutende Kontaminationsgruppe hervorgehoben (Halling-Soerensen et al. 1998). Die Erfassung der Bakterientoxizität von Antibiotika mit Hilfe des normierten Leuchtbakterientestes (z.B. nach DIN 38412-L34 oder ISO 11348) ist jedoch vielfach nur sehr ungenügend möglich (Backhaus et al. 1997). Wie das in Abbildung 3 dargestellte Beispiel exemplarisch für das Antibiotikum Gentamycin zeigt, manifestiert sich die Bakterientoxizität erst bei Gewährung einer verlängerten Auswirkungszeit von 24 h (im Vergleich zu standardisierten 30 min) möglich. Gentamycin ist bekannt als Proteinbiosyntheseinhibitor, der seine Wirkung durch spezifische Bindung an bakterielle 30S rRNA entfaltet. Offensichtlich ist

die Störung der Proteinbiosynthese innerhalb der üblichen Beobachtungszeiträume von 5, 15 und 30 min nicht hinreichend relevant, um einen Effekt auf das Lumineszenzverhalten von *Vibrio fischeri* zu haben. Gleichwohl ist eine sensitive Erfassung dieser ökotoxikologisch höchst relevanten Wirkqualität mit einem verlängertem Testregime sehr wohl möglich.

Abb.3: Inhibition bakterieller Lumineszenz nach 30 min bzw. 24 h durch das



Antibiotikum Gentamycin.

Das Auftreten von Stoffgemischen anstelle von analysenreinen Einzelsubstanzen in der Umwelt ist eine lange anerkannte Realität (Vouk et al. 1987). Die biologische Toxizitätsprüfung von komplex belasteten Abwässern etwa nach dem Wasserhaushaltsgesetz versucht dieser Tatsache Rechnung zu tragen. Aus theoretisch-pharmakologischen Überlegungen zum Zusammenwirken von Stoffen in einer Mischung kann erwartet werden, das Stoffmischungen aus Stoffen die “ähnliche” wirkungsauslösende Ursachen in Organismen haben, sich wie Verdünnungen ein und derselben Substanz verhalten. Ihre Kombina-

tionswirkung ließe sich dann als konzentrationsadditiv kalkulieren (Altenburger et al. 1996). Sofern pharmakokinetisch keine Besonderheiten für die Komponenten einer derartigen Mischung vorliegen, ist hierbei auch keine Zeitabhängigkeit in der Beurteilbarkeit der Toxizität dieser Art von Stoffgemischen zu erwarten.

Anders ist die Situation jedoch für Stoffmischungen aus “unähnlich” wirkenden Substanzen. Hier ist anzunehmen, daß das Konzept der Unabhängigen Wirkung (Independent Action) brauchbare Prognosen zu Kombinationseffekten liefert (Grimme et al. 1996). Wenn Schadstoffe primär mit unterschiedlichen Strukturen in Organismen wechselwirken und demzufolge verschiedenartige biologische Prozesse (z.B. Photosynthese und DNS-Replikation) störend beeinflussen, sind auch unterschiedliche Effektorketten erforderlich, um einen Effekt auf der Ebene des unter Beobachtung stehenden biologischen Parameters (Schwimmfähigkeit, Vermehrung, Energiehaushalt) sichtbar werden zu lassen. Für Mischungen aus derartig verschiedenartigen Stoffen, wie sie in der Umwelt vermutlich eher die Regel, denn die Ausnahme darstellen, ist somit theoretisch zumindest eine Zeitabhängigkeit in der Ausprägung derartiger Mischungstoxizitäten plausibel. Abbildung 4 zeigt anhand des Mischungstoxizitätsvergleiches von Zweistoffgemischen im Kurzzeit-Leuchtbakterientest nach 30 min und Langzeit-Leuchtbakterientest nach 24 h, daß diese Überlegungen nicht nur von theoretischer Bedeutung sind. Dargestellt sind die Verhältnisse von nach Unabhängiger Wirkung erwarteter zu experimentell beobachteter Mischungstoxizität für das EC50-Effektniveau der Leuchtbakterienhemmung. Während im Biotest nach 30 min noch für die Mehrzahl der untersuchten Gemische aus zwei Substanzen unterschiedlicher Wirkung der Kombinationseffekt auf der Basis der Einzelstofftoxizitäten deutlich überschätzt wird, ist nach 24 h das Bild völlig anders. Die Mehrzahl der geprüften Mischungen werden im

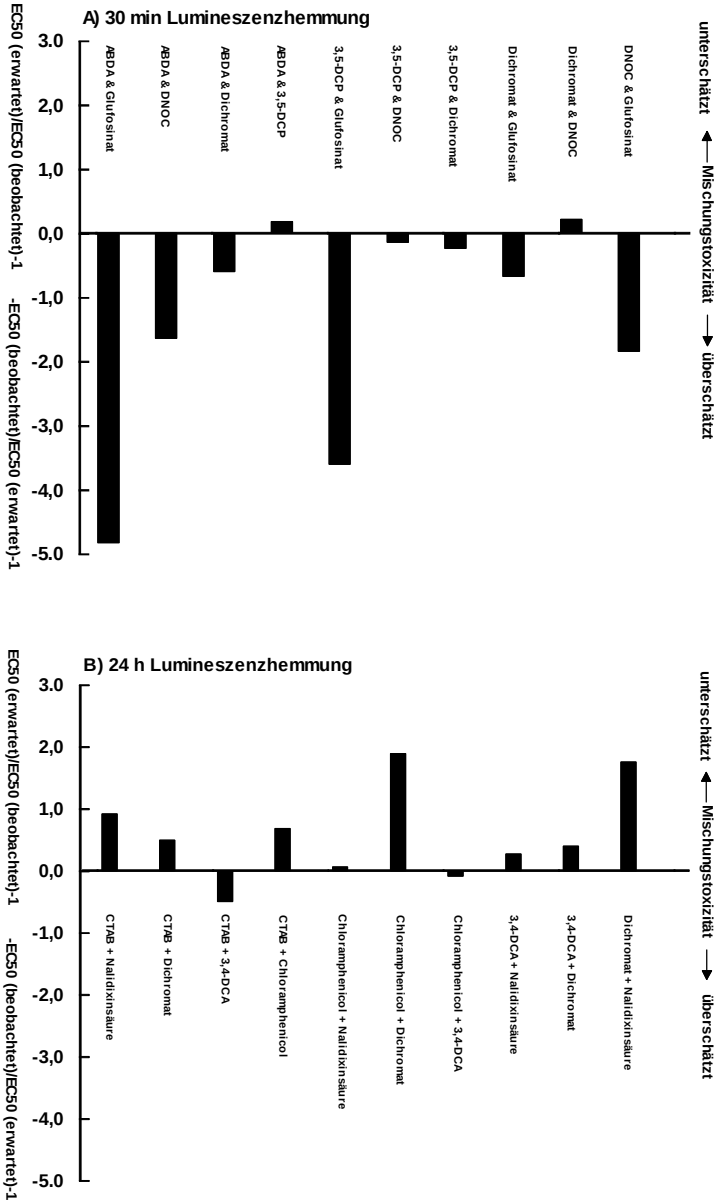


Abb. 4.: Die Stärke von Kombinationseffekten kann bei Mischungen aus Stoffen mit unterschiedlichen Wirkweisen mit der Auswirkungszeit gegenüber der nach Unabhängiger Wirkung erwarteten Mischungstoxizität zunehmen. (Abk.: ABDA Alkylbenzyldimethyl-ammonuimchlorid; DNOC Dinitroorthocreosol; 3,5-DCP 3,5-Dichlorphenol; 3,4-DCA 3,4-Dichloranilin)

Rahmen biologischer Varianz plausibel in ihrem Kombinationseffekt prognostiziert. Die tatsächliche Mischungstoxizität wird maximal um einen Faktor 2 unterschätzt. Dieses Beispiel weißt auf die Notwendigkeit hin, insbesondere in Fällen der Testung komplex belasteter Umweltproben wie sie z.B. viele Abwässer darstellen, die Folgen längerer Auswirkungszeiten zu reflektieren.

Zeit-Wirkungs-Analyse

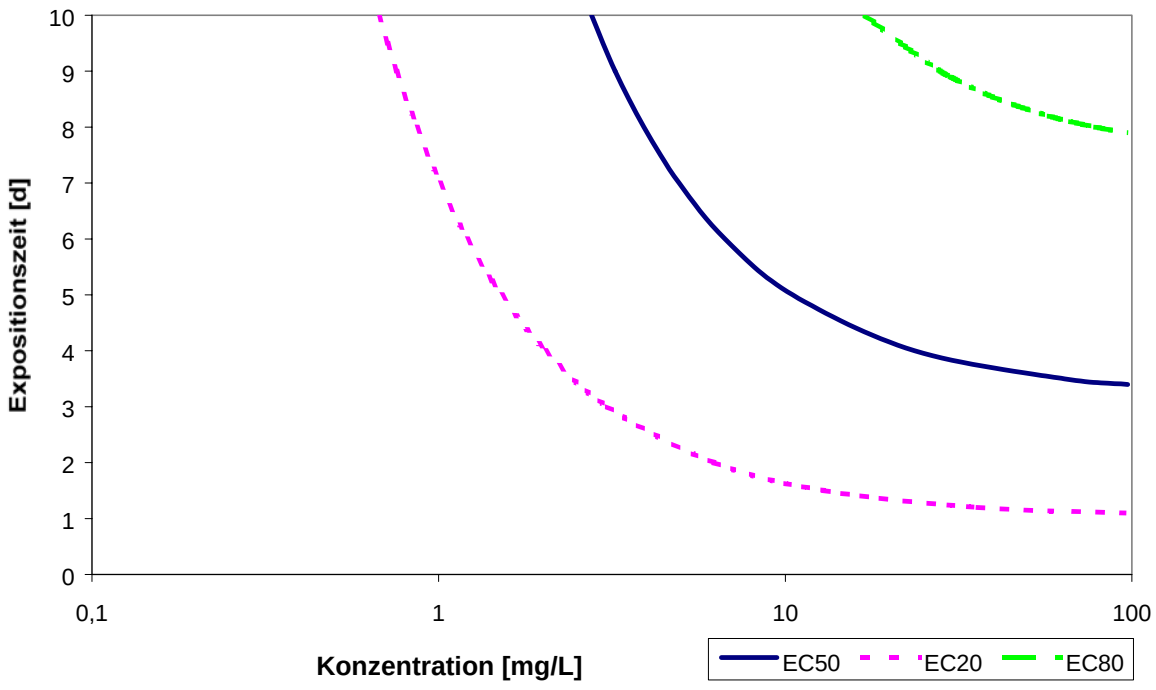
Die bislang besprochenen Probleme einer verläßlichen Beurteilung von toxischen Schadstoffpotentialen mit Hilfe des Einsatzes von Biotesten haben als gemeinsame Ursache, daß in standardisierten Biotesten in der Regel ein mehr oder weniger willkürlich festgelegter Beobachtungszeitpunkt als sogenannter Wirkungsendpunkt für die Beschreibung schädlicher Effekte verwendet wird. Die Gründe für derartige Festlegungen sind vielfältig und einleuchtend:

- Bei gegebenem möglichen Aufwand kann eine Berücksichtigung von unterschiedlichen Beobachtungszeitpunkten nur auf Kosten der Anzahl beobachteter Konzentrations/Verdünnungsabstufungen erfolgen;
- Eine Festlegung auf definierte Beobachtungszeitpunkte schafft eine homogenere Situation für Beurteilungen;
- Die Beurteilung von Zeit-Wirkungs-Beziehungen ist im Vergleich zur Beschreibung von Konzentrations-Wirkungs-Beziehungen weniger entwikkelt.

Das Faktum, daß die Analyse der Wirkung des Testgutes nur selten nach einer biologisch begründeten Expositionsdauer erfolgt, bleibt davon jedoch unberührt. Die Homogenität von Beurteilungen wird mit potentiell gravierenden Fehleinschätzungen zur Toxizität von Stoffen eingekauft. Mit dem in Abbildung 5 dargestellten Beispiel soll darauf hingewiesen werden, daß es mittlerweile die Nutzung von modellhaften Zeit-Wirkungs-Analysen mit Daten aus standardisierten Biotesten möglich ist. Abgebildet sind Kurven gleicher Wirkung in Form geschätzter EC-Werte modelliert nach dem Modell “DebTox” (Kooijman und

Bedeaux, 1996) auf der Basis von in zweitägigen Abstand erfolgten Frondzählungen im Biotest mit der Waserlinse *Lemna minor* unter Einfluß von Kaliumdichromat. Die mittlere Linie etwa kennzeichnet den modellierten Zusammenhang zwischen der Konzentration und der Zeit an der eine Beobachtung stattfindet, bei der eine 50% Hemmung des Populationswachtumes zu erwarten ist. In die Terminologie der Konzentrations-Wirkungs-Beziehungsanalyse übersetzt ergibt sich, daß bei einer wie derzeit von der OECD vorgeschlagenen Beobachtungszeit von 7 Tagen, der EC50 für Kaliumdichromat mit ca. 5 mg/L beziffert werden müßte, während das Modell zeigt, das bis zum 10. Tag sich

Abb.5: Mit dem Modell DebTox (Kooijmann und Bedaux 1996) modellierter



Zusammenhang für gleiche Wirksamkeiten bei variierender Konzentration und zeitlich gestaffelter Effektbeobachtung für das Frondwachstum im Biotest mit *Lemna minor* unter Einfluß von Kaliumdichromat

dieser Wert halbiert hat. Die Nutzung derartiger, modellierter Zeit-Wirkungs-Zusammenhänge würde einerseits die Beurteilung zeitdeterminierter Expositi-

onssituationen aber auch die Abschätzung von Grenzwertbedingungen erlauben.

Schlußfolgerungen und Ausblick

Es wurde versucht zu verdeutlichen, daß die Praxis des Ausblendens der Zeitabhängigkeit von Toxizitäten durch standardisierte Wirkungsendpunkte in Biotesten zu verschiedensten Beurteilungsproblemen führen kann. Die ausgeführten Beispiele lassen jedoch erkennen, wie mit einfachen technischen Modifikationen in der Durchführung von Routinebiotesten und Einbringung von biologischem Wirkungsverständnis toxikologische Beurteilungen verbessert werden können. So unpopulär die Forderung erscheinen mag, ergibt sich aus dem Dargestellten die Notwendigkeit eine rationale Weiterentwicklung von Biotesten im Hinblick auf experimentelle oder modellhafte Beschreibung von Zeit-Wirkungs-Beziehungen anzustreben. Nur eine wissenschaftlich solide Biotestpraxis vermag auch in Zukunft öffentlichen, administrativen, juristischen und politischen Erwartungen nach rationaler Beurteilung von Risiken durch Umweltkontaminationen zu entsprechen.

Literatur

- Altenburger R, Bödeker W, Faust M, Grimme L H 1996. Regulations for combined effects of pollutants: Consequences from risk assessment in aquatic toxicology. Food and Chemical Toxicology 34, 1155-1157.
- Altenburger R, Jung K, Segner H 1999. Ökotoxische Effekte von Bitterfelder Grundwasserproben. In: Weiß H, Daus B, Teutsch G (Hrsg) UFZ-Bericht Nr. 17/1999, S. 48-52.
- Backhaus T, Froehner K, Altenburger R, Grimme L H 1997: Toxicity testing with Vibrio fischeri: A comparison between the long term (24 h) and short term (30 min) bioassay. Chemosphere, 35, 2925-2938.

- Betts K S 1998. Chemical industry pressured to test high-production volume chemicals. Environ Sci Technol 32, 251A.
- Grimme L H, Faust M, Bödeker W, Altenburger R 1996. Aquatic toxicity of chemical substances in combination: Still a matter of controversy. Human and Ecological Risk Assessment 2, 426-433.
- Halling-Soerensen B, Nors Nielson S, Lanzky P F, Ingerslev F, Holten Lützhoft H C, Joergensen S E 1998. Occurence, fate and effects of pharmaceutical substances in the environment – a review. Chemosphere, **1998**, *36*, 357.
- Kooijman, S.A.L.M., Bedaux, J.J.M. (1996) The analysis of aquatic toxicity data. VU University Press, Amsterdam: 149 S.

Kapitel V

Predictability of the toxicity of multiple chemical mixtures to *Vibrio fischeri*: Mixtures composed of similarly acting chemicals

Rolf Altenburger, Thomas Backhaus, Wolfgang Boedeker, Michael Faust, Martin Scholze und L. Horst Grimme
Environmental Toxicology and Chemistry, 19, 2341-2347. (2000)

SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 19, No. 9, pp. 2341–2347, 2000

Printed in the USA
0730-7268/00 $9.00 + .00

Hazard/Risk Assessment

PREDICTABILITY OF THE TOXICITY OF MULTIPLE CHEMICAL MIXTURES TO *VIBRIO FISCHERI*: MIXTURES COMPOSED OF SIMILARLY ACTING CHEMICALS

ROLF ALTENBURGER,*† THOMAS BACKHAUS,‡ WOLFGANG BOEDEKER,‡ MICHAEL FAUST,‡ MARTIN SCHOLZE,‡ and L. HORST GRIMME‡

†Department of Chemical Ecotoxicology, UFZ Centre for Environmental Research Leipzig-Halle, Permoserstrasse 15, 04318 Leipzig, Germany

‡Institute of Cell Biology, Biochemistry, and Biotechnology, University of Bremen, Leobener Strasse, 28334 Bremen, Germany

(*Received* 12 *April* 1999; *Accepted* 13 *January* 2000)

Abstract—The prediction of combined effects based on the effects of the individual components of mixtures by using the pharmacological concepts of concentration addition and independent action might be a promising tool for the risk assessment of pollutant mixtures. To analyze and compare the predictive capabilities of the reference concepts for similarly acting chemicals, the overall toxicity of a multiple mixture was determined in a bioluminescence inhibition assay with *Vibrio fischeri*. The mixture was composed of 16 similarly and specifically acting chemicals, anticipated to have a common mode of action via weak acid respiratory uncoupling of oxidative phosphorylation. Results show that the observed mixture toxicity is rather well predicted by both concepts. Concentration addition shows an excellent predictive power; the median effective concentration (EC50) of the mixture is predicted with an error of about 10%. Independent action, in contrast, underestimates the EC50 of the mixture by a factor of a little more than three. With respect to risk assessment procedures, it may be concluded that concentration addition gives a valid estimation of the overall toxicity for multiple mixtures with similar and specific mechanisms of action of the mixture components in this type of biotest.

Keywords—Concentration addition Independent action Mixture toxicity *Vibrio fischeri* Uncouplers

INTRODUCTION

Chemical risk management procedures commonly rely on effect assessments based on single-substance evaluations. Organisms, however, are typically exposed to multiple mixtures of chemicals. Due to the temporal and spatial variability of the composition of mixtures, a direct assessment of combined effects is not feasible in many cases. If the mixture components are known, a promising alternative is to predict the mixture toxicity from the toxicity of the individual components. Several approaches for the calculation of the expected mixture toxicity have been introduced [1].

In aquatic toxicology, concentration addition is commonly used for the assessment of combination effects. This concept has been introduced by Loewe and Muischnek [2,3] and is based on the idea that the components of a given mixture have a common site of action [4,5]. In the most simple case, concentration addition is said to occur if a chemical acts like a dilution of another, meaning that the effect can be obtained by replacing one chemical totally or in part by the equieffective amount of another chemical. Due to its reasonable pharmacological basis, concentration addition has gained large acceptance and has even been proposed as the general solution for mixture toxicity analysis [1].

Concentration addition is expressed mathematically as

$$\sum_{i=1}^{n} \frac{c_i}{\mathrm{EC}x_i} = 1 \qquad (1)$$

where n is the number of mixture components, $\mathrm{EC}x_i$ is the concentration of the ith mixture component that provokes x% effect when applied singly and c_i is the concentration of the respective component in the mixture. Each fraction ($c_i/\mathrm{EC}x_i$) represents the concentration of a mixture component scaled for its relative toxicity and is generally termed the toxic unit of that component. Consequently, each compound in the mixture can be replaced by another without changing the overall toxicity as long as the sum of toxic units remains unchanged. If, at a total concentration of the mixture provoking x% effect, the sum of the toxic units equals one, concentration addition holds. Otherwise, more or less than (concentration) additive effects are assessed.

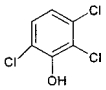
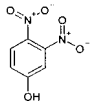
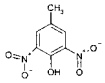
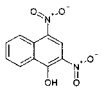
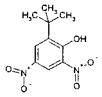
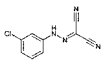
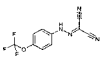
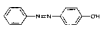
Evidence using aquatic biotests with different species has been produced, demonstrating that concentration addition is well suited for the prediction of the toxicity of multiple mixtures of unspecifically acting substances, mainly organic, nonreactive chemicals with industrial uses and with narcotic properties [6–10]. Moreover, first evidence for binary mixtures of pesticides in an algal biotest show again good predictability of the combined effects using concentration addition [11].

The competing concept of independent action has gained only little attention in aquatic toxicology [12] but is quite popular, e.g., in assessing mutagenic effects for multiple-drug exposure [1]. Also, in risk management recommendations for handling mixture toxicities, this concept is frequently implicitly employed [13]. As the name implies, independent action (also known as response addition, effect multiplication, or Bliss independence) is based on the assumption that the compounds of a given mixture act independently in a statistical sense [4], which, in toxicological terms, is often understood in the sense that compounds having different molecular acceptor sites [14] may act on different physiological systems within the exposed organisms [15]. The mathematical formulation is as follows:

$$\mathrm{E}(c_{\mathrm{Mix}}) = \mathrm{E}(c_1 + \cdots + c_N) = 1 - \prod_{i=1}^{n} [1 - \mathrm{E}(c_i)] \qquad (2)$$

* To whom correspondence may be addressed (ra@uoe.ufz.de).

Table 1. Sixteen phenol derivatives as mixture components

Substance	Structure	Substance	Structure
Chlorophenols			
2,3,4-Trichlorophenol (CAS RN 15950-66-0)		2,3,5-Trichlorophenol (CAS RN 933-78-8)	
2,3,6-Trichlorophenol (CAS RN 933-75-5)		2,4,6-Trichlorophenol (CAS RN 88-06-2)	
Pentachlorophenol (CAS RN 87-86-5)			
Dinitrophenols			
2,4-Dinitrophenol (CAS RN 51-28-5)		2,6-Dinitrophenol (CAS RN 573-56-8)	
3,4-Dinitrophenol (CAS RN 577-71-9)		2,6-Dinitro-4-methylphenol (CAS RN 609-93-8)	
2,4-Dinitro-1-naphtol (CAS RN 605-69-6)		Dinoseb (CAS RN 88-85-7)	
Dinoterb (CAS RN 1420-07-1)		DNOC[a] (CAS RN 534-52-1)	
Others			
CCCP[b] (CAS RN 555-60-2)		FCCP[c] (CAS RN 370-86-5)	
		4-Phenylazophenol (CAS RN 1689-82-3)	

[a] DNOC = 4,6-dinitro-*o*-cresol.
[b] CCCP = carbonyl cyanide-*m*-chlorophenyl-hydrazone.
[c] FCCP = carbonyl cyanide-*p*-trifluoro-methoxyphenyl-hydrazone.

where $E(c_{Mix})$ denotes the predicted effect (scaled to 0–1) of an *n*-compound mixture, c_{Mix} is the total concentration in the mixture, c_i is again the concentration of the *i*th compound, and $E(c_i)$ is the effect of that concentration if the compound is applied singly.

In effect, two valid reference concepts that allow predicting combined effects on the basis of information of the concentration effect relationships of the single components are available. They form the rationale behind most of the methods in the literature that are proposed to assess combined effects and also are a major reason for existing terminological confusion [16,17]. While there is knowledge on the qualitative and quantitative relationships between the predictions resulting from both concepts [18,19], there is no clear understanding of which concept is used most appropriately for what type of mixtures simply due to a dearth of studies that employ both concepts in a comparative manner for mixture toxicity prediction.

Prerequisites for such efforts are an appropriate biometrical design that allows the calculation of combined effects according to both concepts, a substance selection that meets the conceptual premises of a similar type of action of the mixture components, and a precise description of the concentration–response relationships of the single compounds.

The aim of our study was to investigate the predictability of a multiple mixture of similarly acting components with a known specific mode of action using both reference concepts for prediction.

MATERIALS AND METHODS

Test chemicals

All 16 uncouplers of oxidative phosphorylation selected as mixture components were phenol-type compounds and were purchased in the highest available purity from Aldrich (Steinheim, Germany), Riedel (Seelze, Germany), Merck (Duesseldorf, Germany), or Sigma (Deisenhofen, Germany). The components are shown in Table 1. Stock solutions in organic solvents (methanol, pro analysis grade, Merck) were prepared of all chemicals and were stored at −20°C. The stock solutions were used throughout the study. For testing, aliquots were evaporated under N_2 and redissolved in 2% NaCl solution overnight (pH 7.0). These solutions were adjusted to a pH of

6.9 ± 0.2 prior to testing and analysis of nominal concentrations.

Biotest

For the determination of the single as well as the mixture toxicities, we employed the short-term bioluminescence inhibition assay using the marine bacterium *Vibrio fischeri* (formerly *Photobacterium phosphoreum*) according to the protocol of the International Standard Organization (ISO), Geneva, Switzerland, 11348-2 [20], as this biotest is able to produce large amounts of reliable toxicity data in a relatively short period of time. For each mixture component, concentration–response data covering the effect range from >1% to at least 80% effect were generated.

The bacteria were purchased as liquid dried kits (Dr. Lange, GmbH, Duesseldorf, Germany). They were stored at −20°C and rehydrated prior to testing. Testing was conducted using 100 μl bacterial suspension mixed with 500 μl aqueous solution of the respective test chemical(s). The bioluminescence for each sample was measured in a luminometer (Lumistox, Dr. Lange) after an incubation time of 30 min.

Experimental approach

The toxicity of the 16-compound mixture was determined using a fixed ratio design. While keeping the mixture ratio constant, the total concentration of the mixture was varied so that a complete concentration–response relationship of the mixture could be described experimentally. Two different mixture ratios, based on the relative toxicities of the components, were examined. First, a mixture whose components were mixed in the ratio of the EC50s of the individual compounds was analyzed. This is an often-used design that leads to so-called equitoxic mixtures and has been widely used for assessing the combined effect of chemical mixtures (cf., [6]). At a mixture concentration that equals the sum of 1/*n* of the EC50s of the individual components, concentration addition predicts exactly 50% effect of the mixture (see Eqn. 1).

In a second experiment, the components were mixed in the ratio of their EC1 values. The total mixture concentration that equals the sum of the individual EC1 values is the lowest total concentration at which all components are present in concentrations whose effect (if they would have been applied singly) can be calculated using the outlined experimental and biometrical approaches. This experimental design allows a higher discrimination between the mixture toxicity predictions resulting from both concepts.

Toxicity determinations

An identical experimental design was used for the toxicity determinations of the single substances and the mixtures. At least 15 different concentrations were analyzed, each tested in triplicate. The concentrations were chosen to allow for a valid description of the complete range from 1 to at least 80% effect in the case of single substances and from 10 to at least 90% in the case of the mixtures. A minimum of 11 untreated samples served as the controls. The calculation of inhibition of bioluminescence was made according to ISO 11348 [20]. Only the correction factor that controls for the time-dependent change of bioluminescence in the untreated situation has been calculated differently. Checking the 12 controls per experiment at the various positions of the temperature incubation block, it was observed that the luminescence of the bacteria changed already during the period of the initial reading in a time-dependent manner. This was accounted for by using a second-grade polynomial adapted to the controls of the specific experiment instead of a constant correction factor as proposed by the ISO guideline.

1) Probit: $P(Conc) = \frac{1}{2\pi}\int_{-\infty}^{\theta_1+\theta_2\log_{10}(Conc)} \exp(-u^2/2)du = \Phi(\theta_1 + \theta_2 \log_{10}(Conc))$

2) Logit: $P(Conc) = \frac{1}{(1+\exp(-\theta_1 - \theta_2 \log_{10}(Conc))}$

3) Morgan-Mercier: $P(Conc) = 1 - \frac{1}{1+(\theta_1 Conc)^{\theta_2}}$

4) Weibull/Gompertz: $P(Conc) = 1 - \exp(-\exp(\theta_1 + \theta_2 \log_{10}(Conc)))$

5) Generalisied Logit 1: $P(Conc) = \frac{1}{(1+\exp(-\theta_1 - \theta_2 \log_{10}(Conc))^{\theta_3}}$

6) Generalisied Logit 2: $P(Conc) = 1 - \frac{1}{(1+\exp(\theta_1 + \theta_2 \log_{10}(Conc))^{\theta_3}}$

7) Aranda-Ordaz: $P(Conc) = 1 - \frac{1}{(1+\exp(\theta_1 + \theta_2 \log_{10}(Conc))/\theta_3)^{\theta_3}}$

8) Logit with Box-Cox-Transformation:

$$P(Conc) = \left(1+\exp\left(-\theta_1 - \theta_2 \frac{Conc^{\theta_3}-1}{\theta_3}\right)\right)^{-1}$$

9) Weibull with Box-Cox-Transformation:

$$P(Conc) = 1 - \exp\left(-\exp\left(\theta_1 + \theta_2 \frac{Conc^{\theta_3}-1}{\theta_3}\right)\right)$$

10) Probit with Box-Cox-Transformation:

$$P(Conc) = \Phi\left(\theta_1 + \theta_2 \frac{Conc^{\theta_3}-1}{\theta_3}\right)$$

whereby Φ(y) is the cumulative normal (Gauss) distribution, meaning that the probability that a standard normal random variable is less than y, P(Conc) is the mean effect of the model, θ1, θ2, θ3 are the (unknown) model parameters to be estimated, and Conc denotes the concentration of the tested substance.

Fig. 1. Regression models used to calculate the concentration–response relationships.

Chemical analysis

In order to check whether the concentrations of the test chemicals were achieved in the applied stock solutions, high-performance liquid chromatography analyses were carried out using reversed phase (C18) endcapped columns, a mobile phase consisting of 40% acetonitrile and 60% H_3PO_4 (0.01%), and a compound-specific ultraviolet detection wavelength (between 250 and 360 nm). A check on stability was carried out exemplarily for 4,6-dinitro-*o*-cresol, 2,4-dinitrophenol, and carbonyl cyanide-*m*-chlorophenyl-hydrazone. Within the 30 min of incubation at 15°C, concentrations were found to remain within 98 to 101% of the initial concentration. All concentrations provided are based on the validated nominal concentrations of the aqueous stock solutions.

Data analysis

The concentration–response relationships of the single substances as well as of the mixtures were calculated using a best fit approach [21] and will be the subject of a separate publication. For that purpose, 10 different two- or three-parametric sigmoidal models, including the commonly used probit, logit, and Weibull models, were selected (Fig. 1). All models are confined to the range >0 to <100% effect. The parameters of the models were estimated using a generalized least-square approach [22]. For each individual set of data, the best fitting model (based on the sum of absolute errors and an analysis of residues) was chosen. The statistical uncertainty of the calculated effect concentrations was estimated using the bootstrap approach [21,23,24].

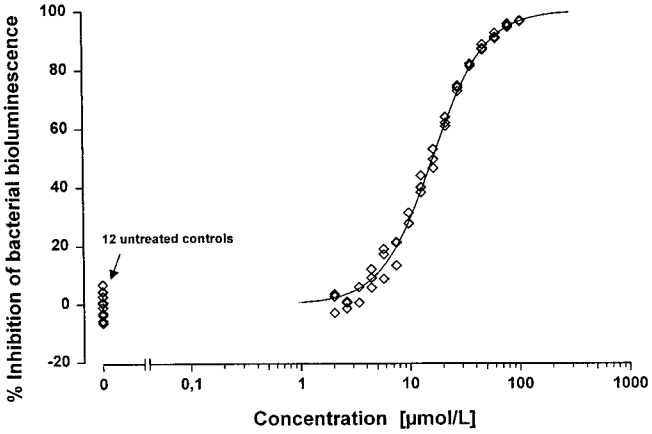


Fig. 2. Experimental effect data and fitted concentration–response function for dinoseb.

Prediction of mixture toxicities

As the composition of the mixture is quantitatively known, the concentration of each component can be expressed as a fraction of the total concentration. Consequently, for the calculation of the effect concentrations predicted by concentration addition, Equation 1 can be rewritten as

$$\mathrm{EC}x_{\mathrm{Mix}} = \left(\sum_{i=1}^{n} \frac{p_i}{\mathrm{EC}x_i}\right)^{-1} \quad (3)$$

where $\mathrm{EC}x_{\mathrm{Mix}}$ is the total concentration of the mixture provoking x% effect and p_i denotes the fraction of component i in the mixture. Using Equation 3, the total concentrations of each mixture giving 1 to 99% effect were calculated in steps of 1%. The resulting 99 concentration/effect pairs were connected by straight lines, providing a visualization of the predicted concentration–response curve.

On the basis of the total concentration and a known ratio of the components, Equation 2 allows the calculation of mixture effects according to independent action. These were estimated using the concentrations calculated for concentration addition according to the procedure described in Backhaus et al. [25].

RESULTS

Single-substance toxicity

The quality of data generated for the estimation of concentration–response functions for the single compounds and the fit of the best fitting regression model (Box–Cox–logit) are shown in Figure 2. It can been seen that variance of the observed effects is higher in the low effect range but still very small for a biological test system. The toxicity parameters of all 16 selected mixture components are summarized in Table 2, and the corresponding concentration–response functions are visualized regarding shape and position in Figure 3. The EC50 values span approximately 2.5 orders of magnitude, ranging from 0.57 µmol/L for carbonyl cyanide-*p*-trifluoro-methoxyphenyl-hydrazone to 91 µmol/L for trichlorophenol. As it can be seen in Figure 3, the shapes of the concentration–response curves are relatively similar, with the exceptions of 4-phenylazophenol and 2,4-dinitrophenol.

Only for 1 out of the 16 chemicals was the concentration–response relationship calculated with a model typically used in aquatic toxicology (Weibull). In all other cases, various models, especially the Box–Cox transformations of probit, logit, and Weibull models, proved to be superior.

Mixture toxicity

The results of the experimental determination of the mixture toxicities as well as the predictions made by the reference concepts, concentration addition and independent action, are depicted

Table 2. Single-substance toxicity of mixture components and their mixture toxicities; θ_i denotes the parameter of the concentration response function (see Fig. 1); the 95% confidence interval is given in brackets; components are listed with increasing median effective concentration EC50[a]

	Fit	θ_1	θ_2	θ_3	EC1 (µmol/L)	EC50 (µmol/L)
Toxicities of the single mixture components						
Mixture component						
FCCP	Box–Cox–Probit	0.73	1.29	0.016	0.09 [0.05–0.14]	0.57 [0.49–0.65]
CCCP	Box–Cox–Probit	0.25	1.20	0.054	0.10 [0.06–0.15]	0.81 [0.74–0.89]
4-Phenylazophenol	Box–Cox–Weibull	−2.08	0.78	−0.033	0.05 [0.01–0.13]	9.88 [9.15–10.67]
Dinoterb	Generalized logit I	−7.71	6.62	0.706	1.51 [0.96–2.36]	12.20 [11.06–13.25]
Pentachlorophenol	Box–Cox–probit	−4.88	2.34	−0.167	3.34 [2.44–4.31]	12.98 [12.29–13.84]
2,4-Dinitro-1-naphtol	box–Cox–Weibull	−9.79	5.29	−0.340	3.30 [1.83–4.68]	15.44 [14.57–16.40]
Dinoseb	Box–Cox–logit	−5.05	1.79	0.005	1.29 [0.88–1.80]	16.40 [15.84–16.99]
2,3,4-Trichlorophenol	Box–Cox–Weibull	−6.12	2.31	−0.095	1.98 [1.01–3.30]	17.25 [16.43–18.01]
2,3,5–Trichlorophenol	Box–Cox–Weibull	−38.32	31.61	−0.738	8.15 [6.34–9.69]	19.05 [18.66–19.46]
2,4,6–Trichlorophenol	Box–Cox–Probit	−2.57	0.94	−0.098	1.30 [0.60– 2.20]	24.38 [22.13–26.71]
2,6-Dinitro-4-methylphenol	Box–Cox–Probit	−2.31	0.65	0.030	0.98 [0.45–1.61]	29.76 [27.55–32.44]
2,4-Dinitrophenol	Box–Cox–Probit	−1.31	0.35	0.009	0.05 [0.01–0.14]	39.24 [35.64–43.42]
DNOC	Box–Cox–Probit	−3.06	0.59	0.111	3.20 [0.85–7.18]	58.95 [54.16–63.16]
2,6-Dinitrophenol	Box–Cox–Probit	−4.48	1.22	−0.084	6.78 [3.92–11.0]	81.10 [73.49–88.43]
3,4-Dinitrophenol	Weibull	−6.18	2.99		3.38 [2.15–5.31]	87.54 [78.83–95.91]
2,3,6-Trichlorophenol	Box–Cox–Weibull	−4.82	1.10	−0.047	1.23 [0.12–4.53]	90.6 [78.9–104.30]
Toxicity of the mixture (16 components)						
Mixture ratio						
EC50	Generalized logit I	−42.82	23.73	0.0835		28.50 [27.88–29.09]
EC01	Box–Cox–probit	−2.38	0.221	0.7445		19.17 [18.93–19.41]

[a] EC1 = concentrations estimated to provoke an effect of 1%; FCCP = carbonyl cyanide-*p*-trifluoro-methoxyphenyl-hydrazone; CCCP = carbonyl cyanide-*m*-chlorophenyl-hydrazone; DNOC = 4,6-dinitro-*o*-cresol.

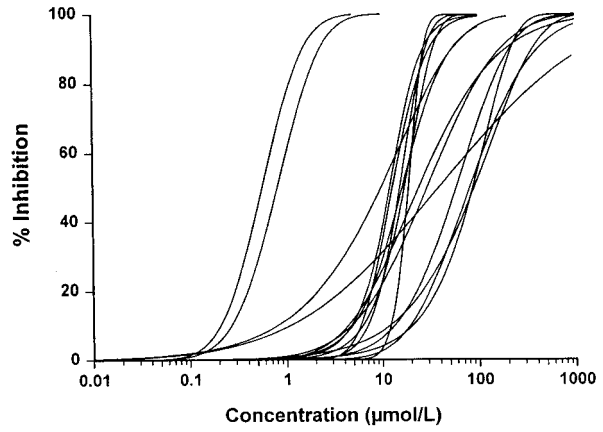


Fig. 3. Concentration–response curves of all 16 mixture components; components may be identified from left to right using Table 2.

in Figure 4. For both mixture ratios tested, we obtained data that permitted a valid calculation of concentration–response relationships, which are shown in Table 2. The EC50 values determined for both tested mixture ratios (28.5 µmol/L for a ratio of the EC50 of the individual compounds and 19.2 µmol/L for the EC1 ratio) are lower than the EC50 of the least toxic component and higher than the EC50 of the most toxic component. Regarding the possibility that only the most toxic substance dominates the mixture effect (as assumed for the specific case of completely negatively correlated susceptibilities of responses, also called no addition) [6], it is easy to see that, for both mixtures studied here, the expected contribution of carbonyl cyanide-*p*-trifluoro-methoxyphenyl-hydrazone as the single most potent component regarding its concentration at the EC50 of the mixture is well below 1% effect.

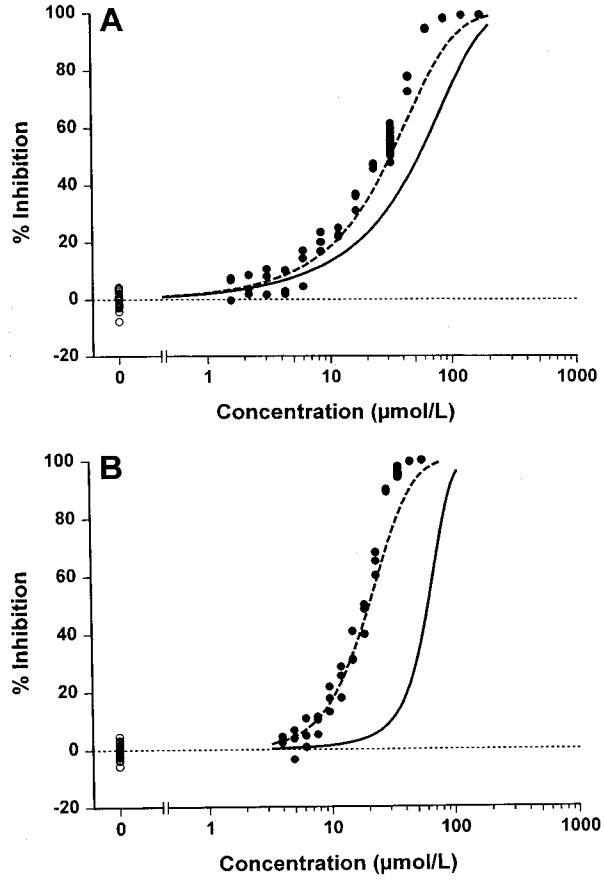


Fig. 4. Predicted and observed mixture toxicity. (**A**) Mixture ratio derived from EC50s of the individual components; (**B**) mixture ratio derived from EC1s of the individual components. – – –, Prediction according to concentration addition; ———, prediction according to independent action.

As can be seen from Figure 4, concentration addition predicts a higher mixture toxicity than the competing concept of independent action. This is not specific for the mixture ratio analyzed and the effect level under observation. Figure 4 also shows that the extent of the difference between the predictions is dependent on the mixture ratio and the effect level. If the components are mixed in the ratio of their EC50s, there is only a factor of 1.7 between the EC50 predicted by concentration addition (32.2 µmol/L) and independent action (55.2 µmol/L). Mixing the compounds in their EC1 ratio gives a factor of 3.0 between the two predicted EC50 values (concentration addition, 20.6 µmol/L; independent action, 62.1 µmol/L).

Indifferent to the mixture ratio and the effect level, the mixture toxicity is rather precisely predicted by concentration addition. If the components are mixed in the ratio of their respective EC50s, the observed EC50 is 28.5 µmol/L, which is only a difference of 13% with respect to the predicted value of 32.3 µmol/L. The excellent predictive power of concentration addition becomes even more prominent for the EC1 mixture. The EC50 predicted by concentration addition is 20.6 µmol/L, the observed EC50 is 19.2 µmol/L—a difference of 7.5%. Only in the higher effect regions of the EC50 and the EC1 mixtures are there small differences between observations and predictions for concentration addition (at maximum, a factor of 1.7). In contrast, the concept of independent action underestimates the mixture toxicity at every response level.

DISCUSSION

Single-substance toxicities

The toxicities of 16 protonophoric uncouplers of the oxidative ADP-phosphorylation, nitroanilines and nitrophenols, to the marine bacterium *V. fischeri* have been determined by Schultz and Cronin [26] using the Microtox® assay (Azur Environmental, Carlsbad, CA, USA). Four of the compounds, namely 2,4-dinitrophenol, 2,6-dinitro-4-methylphenol, 4-phenylazophenol, and pentachlorophenol, have also been studied in this paper. The comparison of estimated EC50 values (Table 3) shows that, with the exception of pentachlorophenol, there is good confirmation of the previous experimental results. Moreover, using the quantitative structure–activity relationships (QSAR) proposed by Schultz and Cronin for an uncoupling effect in *V. fischeri,* it can been seen from Table 3 that the ratio between calculated and determined EC50 values of this study indeed shows good agreement between determined toxicity for the compounds used in this study and the expected toxicity based on the QSAR approach. The few exceptions comprise carbonyl cyanide-*m*-chlorophenyl-hydrazone and carbonyl cyanide-*p*-trifluoro-methoxyphenyl-hydrazone, with a clearly higher toxicity than derived from the nitroanilines and nitrophenols employed in formulating the QSAR equation. The 2,3,6-trichlorophenol and pentachlorophenol, on the other hand, are clearly less toxic than expected on the basis of the QSAR. The lower toxicity for pentachlorophenol may not be attributed to solubility problems as we have checked analytically the nominal concentrations.

Table 3. Bacterial toxicities of phenolic compounds compared with quantitative structure–activity relationships (QSAR)-based predictions[a]

Compound	MW	log K_{ow}[b]	Experimental data[c] EC50 (mg/L)	QSAR for uncoupling[d] (mg/L)	QSAR for minimum toxicity[e] (mg/L)	Results of this study EC50 (mg/L)	Results of this study EC50 (μmol/L)	TR uncoupling toxicity/observed EC50	TR minimum toxicity/observed EC50
2,3,4-Trichlorophenol	197.45	3.45		3.04	10.06	3.406	17.25	0.89	2.95
2,3,5-Trichlorophenol	197.45	3.45		3.04	10.06	3.761	19.05	0.81	2.67
2,3,6-Trichlorophenol	197.45	3.45		3.04	10.06	17.89	90.59	0.17	0.56
2,4,6-Trichlorophenol	197.45	3.45		3.04	10.06	4.814	24.38	0.63	2.09
2,4-Dinitro-1-naphtol	234.17	2.90		6.69	42.08	3.615	15.44	1.85	11.64
2,4-Dinitrophenol	184.11	1.73	11.0	19.64	482.76	7.224	39.24	2.72	66.83
2,6-Dinitro-4-methylphenol	198.14	2.27	9.05	11.51	150.77	5.896	29.76	1.95	25.57
2,6-Dinitrophenol	184.11	1.73		19.64	482.76	14.93	81.10	1.32	32.33
3,4-Dinitrophenol	184.11	1.73		19.64	482.76	16.12	87.54	1.22	29.95
4-Phenylazophenol	198.23	3.63	0.93	2.49	6.69	1.959	9.88	1.27	3.42
CCCP[f]	204.62	3.75		2.24	5.24	0.166	0.813	13.47	31.51
Dinoseb	240.22	3.67		2.88	7.40	3.940	16.40	0.73	1.88
Dinoterb	240.22	3.64		2.98	7.92	2.930	12.20	1.02	2.70
DNOC	198.14	2.27		11.51	150.77	11.68	58.95	0.99	12.91
FCCP[g]	254.17	4.15		1.78	2.61	0.116	0.567	15.33	22.48
Pentachlorophenol	266.34	4.74	0.52	0.96	0.71	3.458	12.98	0.28	0.21

[a] TR = toxic ratio for expected/observed toxicity; CCCP = carbonyl cyanide-*m*-chlorophenyl-hydrazone; FCCP = carbonyl cyanide-*p*-trifluoromethoxyphenyl-hydrazone; DNOC = 4,6-dinitro-*o*-cresol.
[b] Calculated using KOWWIN, Version 1.54 according to [37].
[c] Experimental results using Microtox test [26].
[d] QSAR model for uncouplers: $1/\log pT30\ [mmol/L] = 0.489 \cdot \log K_{ow} + 0.126$ [26].
[e] Minimum toxicity model: $\log(1/EC50\ [mmol/L]) = 0.995 \cdot \log K_{ow} - 2.14$ [28].

The notion that there is uncoupling of oxidative phosphorylation present as a specific mechanism of action of chemicals in procaryotic cells has been challenged [27], and it is beyond the scope of this article to discuss the physiological reasoning in more detail. However, relating the toxicities determined here for the substituted phenols to a QSAR derived from the experimental study of 22 nonreactive organic chemicals thought to act unspecifically and comparably with the action of anesthetics [28] (Table 3) reveals that indeed most of the compounds employed, again with the exceptions of 2,3,6-trichlorophenol and pentachlorophenol, are more toxic than would be expected for a pure narcotic-type toxicity of the compounds. This can be taken as an indication of a specific toxic activity as opposed to a mere baseline toxic effect. Regarding the outliers, it might be worthwhile to consider to role of ionization in explaining the deviation from a purely log K_{ow}-derived toxicity estimation [29].

Adapting the criteria outlined in European Economic Community Guideline 93/21/EEC [30] to the toxicities shown in Table 2, 3 out of the 16 chemicals tested have to be classified as being very toxic to aquatic organisms and four would be labeled as toxic to aquatic organisms. The components are used as herbicides or industrial chemicals and some are regarded as priority pollutants for aquatic systems.

Predictability of mixture toxicities

The predictive value of concentration addition is clearly demonstrated in Figure 3. The striking predictive power of concentration addition, which is not restricted to a certain mixture ratio or an effect level, strongly supports the notion that the toxicity of multiple mixtures of specifically acting chemicals with similar mechanisms of action may quite precisely be described by the concept of concentration addition.

Hermens and coworkers [28] determined the toxicity of a mixture of 21 nonreactive organic chemicals using the Microtox assay. Interestingly, they found an overestimation by a factor of two for the observed mixture toxicity when using concentration addition to calculate expected combined effects on the basis of the EC50 values for the single components. They suggested that limited bioavailability for very lipophilic compounds could be the reason for deviation from the additivity expectation in this short-term biotest. This might explain the unexpectedly low toxicity of pentachlorophenol observed in this study. But there is no reason to assume any influence of limited bioavailability on the mixture toxicity, as the effects of all compounds are highly reproducible in this study throughout all different effect levels (see Table 2). In contrast, Chen and Chiou [31] described concentration additive behavior of binary mixtures of nonreactive organic chemicals using the Microtox test. However, they restricted the scope of their results to cases of parallel concentration–response curves, providing evidence that compounds with different slopes for their concentration–response curves show systematic deviation from concentration additive behavior [31,32]. The slopes observed in this study are rather comparable, with only two exceptions; however, parallelism is a difficult criterion.

Nirmalakhandan and coworkers [33] reported on the combined effects of multiple-components mixtures on the oxygen uptake of a mixed bacterial population after 6 h of exposure. For different mixtures derived from 50 nonreactive, organic chemicals, they found concentration additive responses irrespective of the method used for mixture toxicity assessment. Thomulka and Lange [34,35] reported on combined effects of binary mixtures of organotin compounds using a luminescence assay with *V. harveyi*. Apparently, here the assessment of the observed mixture toxicities as being in accordance with concentration addition seemed to depend on the method of assessment, in particular on the estimation of variabilities of responses. For the multiple mixtures investigated in this paper, the overlap of prediction and observation is so striking that there seems no reason to ask for statistical confirmation of the result, which indeed would be far from trivial to calculate in

a meaningful way. One might indeed consider the hypothesis by Warne and Hawker [36] that higher numbers of components in a mixture lead to less deviation from concentration additive behavior. The hypothesis has been derived based on a critical volume hypothesis for compounds being present in a biological membrane disturbing their function; however, it has been restricted to nonreactive toxicants.

Acknowledgement—Excellent technical assistance of Carsten Krumbholz is gratefully acknowledged. The study was sponsored by the German Ministry of Education, Science, Research, and Technology project 07 OTX 16.

REFERENCES

1. Berenbaum MC. 1985. The expected effect of a combination of agents: The general solution. *J Theor Biol* 114:413–431.
2. Loewe S, Muischnek H. 1926. Über Kombinationswirkungen. 1. Mitteilung: Hilfsmittel der Fragestellung. *Arch Exp Pathol Pharmakol* 114:313–326.
3. Loewe S. 1927. Die Mischarznei. Versuch einer allgemeinen Pharmakologie der Arzneikombinationen. *Klin Wochenschr* 6: 1077–1085.
4. Bliss CI. 1939. The toxicity of poisons applied jointly. *Ann J Appl Biol* 26:585–615.
5. Plackett RL, Hewlett PS. 1963. Quantal response to mixtures of poisons. *J R Statist Soc B* 14:141–163.
6. Könemann H. 1981. Fish toxicity tests with mixtures of more than two chemicals: A proposal for a quantitative approach and experimental results. *Toxicology* 19:229–238.
7. Hermens J, Canton H, Janssen P, Jong R. 1984. Quantitative structure–activity relationships and toxicity studies of mixtures of chemicals with anaesthetic potency: Acute lethal and sublethal toxicity to *Daphnia magna*. *Aquat Toxicol* 5:143–154.
8. Hermens J, Leeuwangh P, Musch A. 1984. Quantitative structure–activity relationships and mixture toxicity studies of chloro- and alkylanilines at an acute lethal toxicity level to the guppy (*Poecilia reticulata*). *Ecotoxicol Environ Saf* 8:388–394.
9. Könemann H. 1980. Structure–activity relationships and additivity in fish toxicities of environmental pollutants. *Ecotoxicol Environ Saf* 4:415–421.
10. Nirmalakhandan N, Xu S, Trevizo C, Brennan R, Peace J. 1997. Additivity in microbial toxicity of nonuniform mixtures of organic chemicals. *Ecotoxicol Environ Saf* 37:97–102.
11. Faust M, Altenburger R, Boedeker W, Grimme LH. 1994. Algal toxicity of binary combinations of pesticides. *Bull Environ Contam Toxicol* 53:134–141.
12. Stratton GW. 1983. Interaction effects of permethrin and atrazine combinations towards several nontarget microorganisms. *Bull Environ Contam Toxicol* 31:297–303.
13. European Inland Fisheries Advisory Commission. 1987. Water quality criteria for European freshwater fish. Revised report on combined effects on freshwater fish and other aquatic life of mixtures of toxicants in water. EIFAC/T37 Rev 1. Technical Paper. Rome, Italy.
14. Plackett RL, Hewlett PS. 1967. A comparison of models for quantal responses to mixtures of drugs. *Biometrics* 23:27–44.
15. Pöch G. 1993. *Combined Effects of Drugs and Toxic Agents. Modern Evaluation in Theory and Practice.* Springer Verlag, New York, NY, USA.
16. Bödeker W, Altenburger R, Faust M, Grimme LH. 1990. Methods for the assessment of mixtures of pesticides: Mathematical analysis of combination effects in phytopharmacology and ecotoxicology. *Nachrichtenbl Deut Pflanzenschutzdienst* (Braunschweig) 42:70–78.
17. Altenburger R, Bödeker W, Faust M, Grimme LH. 1993. Aquatic toxicology, analysis of combination effects. In Corn M, ed, *Handbook of Hazardous Materials.* Academic, New York, NY, USA, pp 15–27.
18. Boedeker W, Drescher K, Altenburger R, Faust M, Grimme LH. 1993. Combined effects of toxicants: The need and soundness of assessment approaches in ecotoxicology. *Sci Total Environ* Suppl:931–938.
19. Drescher K, Boedeker W. 1995. Assessment of the combined effects of substances: The relationship between concentration addition and independent action. *Biometrics* 51:716–730.
20. International Standards Organization. 1994. Water quality—Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (luminescent bacteria test). ISO 11348-2. Geneva, Switzerland.
21. Grimme LH, Altenburger R, Backhaus T, Boedeker W, Faust M, Scholze M. 1998. *Predictability and Assessment of the Aquatic Toxicity of Mixtures of Substances—Multi-Component Mixtures of Dissimilarly Acting Chemicals at Low Effect Concentrations* (in German). UFZ-Umweltforschungszentrum Leipzig-Halle, Leipzig, Germany.
22. Carroll RJ, Ruppert D. 1988. *Transformation and Weighting in Regression.* Chapman & Hall, New York, USA.
23. Efron B, Tibshirani R. 1993. *An Introduction to Bootstrap.* Chapman & Hall, London, UK.
24. Rosen O, Cohen A. 1995. Constructing a bootstrap confidence interval for the unknown concentration in radioimmunoassay. *Stat Med* 14:935–952.
25. Backhaus T, Altenburger R, Boedeker W, Faust M, Scholze M, Grimme LH. 2000. Predictability of the toxicity of a multiple mixture of dissimilarly acting chemicals to *Vibrio fischeri*. *Environ Toxicol Chem* 19:2348–2356.
26. Schultz TW, Cronin MTD. 1997. Quantitative structure–activity relationships for weak acid respiratory uncouplers to *Vibrio fischeri*. *Environ Toxicol Chem* 16:357–360.
27. Jaworska JS, Schultz TW. 1994. Mechanism-based comparisons of acute toxicities elicited by industrial organic chemicals in procaryotic and eucaryotic systems. *Ecotoxicol Environ Saf* 29:200–213.
28. Hermens J, Busser F, Leeuwangh P, Musch A. 1985. Quantitative structure–activity relationships and mixture toxicity of organic chemicals in *Photobacterium phosphoreum*: The Microtox® test. *Ecotoxicol Environ Saf* 9:17–25.
29. Schultz TW, Bearden AP, Jaworska JS. 1994. A novel approach for estimating toxicity of phenols. *SAR QSAR Environ Res* 5:99–112.
30. Commission of the European Union. 1993. Commission Directive 93/21/EEC of 27 April 1993 adapting to technical progress for the 18th time Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances. *Off J Eur Commun* L-110:0020–0021.
31. Chen C-Y, Chiou Y-S. 1995. Toxicity of binary mixtures of organic chemicals. *Environ Toxicol Water Qual* 10:97–106.
32. Chen C-Y, Huang C-F. 1996. Toxicity of organic mixtures containing cyanogenic toxicants. *Environ Toxicol Chem* 15:1464–1469.
33. Nirmalakhandan N, Arulgnanendran V, Mohsin M, Sun B, Cadena F. 1994. Toxicity of mixtures of organic chemicals to microorganisms. *Water Res* 28:543–551.
34. Thomulka KW, Lange JH. 1995. Multiple toxicity of three metals, tributyltin chloride, dibutyltin dichloride and monobutyltin trichloride, using the marine bacterium *Vibrio harveyi* as the test organism. *Fresnius Environ Bull* 4:508–513.
35. Thomulka KW, Lange JH. 1996. A mixture toxicity study employing combinations of tributyltin chloride, dibutyltin dichloride, and tin chloride, using the marine bacterium *Vibrio harveyi* as the test organism. *Ecotoxicol Environ Saf* 34:76–84.
36. Warne MSJ, Hawker DW. 1995. The number of components in a mixture determines whether synergistic and antagonistic or additive toxicity predominate: The funnel hypothesis. *Ecotoxicol Environ Saf* 31:23–28.
37. Meylan WM, Howard PH. 1995. KOWWIN, Vers. 1.54: Atom/fragment contribution method for estimating octanol-water partition coefficients. *J Pharm Sci* 84:83–92.

Kapitel VI

Quantitative structure-activity analysis of the algal toxicity of nitroaromatic compounds

Heike Schmitt, Rolf Altenburger, Bernd Jastorff und Gerrit Schüürmann
Chemical Research in Toxicology, 13, 441-450. (2000)

Quantitative Structure-Activity Analysis of the Algae Toxicity of Nitroaromatic Compounds

Heike Schmitt,[§] Rolf Altenburger,[§] Bernd Jastorff,[‡] and Gerrit Schüürmann[§*]

[§] Department of Chemical Ecotoxicology
UFZ Centre for Environmental Research
Permoserstr. 15
04318 Leipzig
Germany

[‡] UFT Centre for Environmental Research and Technology
University of Bremen
Leobener Strasse
28334 Bremen
Germany

Corresponding author Gerrit Schüürmann:
Tel ++49-341-235-2309, Fax ++49-341-235-2401, E-mail gs@uoe.ufz.de

Manuscript submitted to *Chem. Res. Toxicol.* on 3 September 1999
Revised manuscript submitted on 1 March 2000

Proliferation toxicity towards the algae *Scenedesmus vacuolatus* in a 24 h one-generation reproduction assay was determined for nitrobenzene and 18 derivatives including two phenols. The resultant EC50 values covering more than four orders of magnitude were subjected to a quantitative structure-activity analysis (QSAR) using hydrophobicity in terms of the octanol/water partition coefficient in logarithmic form, log K_{ow}, and 16 quantum chemical descriptors of molecular reactivity that were calculated with the AM1 scheme. For 13 mononitro derivatives and the highly hydrophobic trifluralin, a narcotic-type mode of action can explain most of the toxicity variation. Correction of log K_{ow} for ionization for the phenols and quantification of the molecular susceptibility for one-electron reduction as apparently rate-determining biotransformation step by the energy of the lowest unoccupied molecular orbital, E_{LUMO}, yields a highly significant QSAR for all 19 compounds (r^2_{adj}=0.90), which can be further improved to r^2_{adj}=0.94 when adding the maximum net atomic charge at the nitro nitrogen, $q_{nitro\text{-}N}$, as third descriptor (r^2_{adj}=0.94). Comparison of the energy of the singly occupied molecular orbital, E_{SOMO}, of the radical anions as initial metabolites with the E_{SOMO} of known redox cyclers suggests that dinitrobenzenes and TFM as well as multiply chlorinated nitrobenzenes may also exert oxidative stress. This is based on an E_{SOMO} window of -0.30 to 0.55 eV as tentative criterion for molecular structures to have the potential for redox cycling, derived from a set of eight known redox cyclers. The discussion includes a detailed analysis of apparently relevant metabolic pathways and associated modes of toxic action of nitroaromatics.

Introduction

Nitroaromatic compounds form an important class of industrial chemicals with substantial marketing volumes and a diverse range of use patterns (*1*). Besides applications as solvents, they are used for the synthesis of dyestuffs, urethan polymers and other plastics as well as of anilines, and among derivative products are also insecticides, herbicides and pharmaceuticals.

Other sources of nitroarenes in the environment include their generation from fuel combustion in vehicles and power plants, and they are also formed as secondary pollutants from reactions with hydroxyl radical ($OH^{\cdot}$) and nitrate radical ($NO_3^{\cdot}$) in the atmosphere under natural NO_x conditions (*2-4*). The latter is of ongoing interest also in order to better understand the mutagenicity of ambient air, pointing to an important aspect of the toxicological profile of this compound class (*4*, *5*). Moreover, there is evidence that nitrophenols are formed photochemically from aromatic precursor compounds in rain, which was discussed in the context of xenobiotics contributing to forest decline (*6*).

From the chemical viewpoint, the nitro group is a strong π-electron acceptor, lowering the electron density of the aromatic ring. Inside the nitro group, excess electronic charge is mainly localized at the oxygen atoms, while the nitrogen atom is typically electron-deficient. As a consequence, nitroaromatic compounds show enhanced reactivity for the attack of nucleophiles at aromatic ring carbons as well as for reactions with reducing agents, and in phenol derivatives the nitro substituent leads to a pronounced enhancement of the acidity of the OH group.

The presence of nitroaromatics in aquatic systems has lead to various studies of the associated hazard potential. Many studies have

concentrated on investigating the acute toxicity towards fish (*7*-*12*) and other aquatic species (*12*-*19*), including the analysis of quantitative structure-activity relationships (QSARs) in order to elucidate underlying modes of action and their link to characteristics of the molecular structure of the compounds.

In such QSAR studies, hydrophobicity as quantified by the decadic logarithm of the octanol/water partition coefficient of the compound, log K_{ow}, is typically used to model baseline toxicity caused by unspecific membrane irritation (*20*, *21*). Excess toxicity of bioreactive compounds can be identified by its upward deviation from hydrophobicity-based QSARs, and there are various molecular descriptors available to model the reactivity profile of the chemicals, which allows a mechanistic interpretation of specific toxicity effects as related to metabolic pathways and chemical interactions with endogenous macromolecules (*20*, *21*).

Nitrobenzene derivatives often show acute toxicities above the baseline toxicity towards aquatic organisms, which has typically been explained by the electrophilic nature of the chemicals (*7*-*9*, *11*, *15*-*17*). Both the ease of reduction to radical anions and subsequent metabolites (*7*, *8*) and electrophilic reactions with electron-rich sites of macromolecules (*9*, *15*, *17*) were considered as a mechanistic basis for the observed excess toxicities, and the preferred electronic parameters were half-wave reduction potential (*8*), Hammett constant σ^- (*8*, *9*, *11*, *13*, *16*), the molecular energy of the lowest unoccupied molecular orbital, E_{LUMO} (*10*, *11*, *16*, *17*) and the acceptor delocalizability (*17*). Particularly enhanced toxicities were observed for dinitrobenzenes, suggesting that there was no commonality in the bioreactivity of mono- and dinitro derivatives (*8*, *13*, *17*).

Comparatively few investigations have concentrated on oxidative stress via redox cycling as another mode of toxic action towards aquatic species (*22*-*26*). Besides known redox cyclers such as quinones like tetramethylbenzoquinone (*25*) and nitrofuran derivatives such as nitrofurantoin (*23*, *24*), redox cycling activity has also been demonstrated for the nitroaromatic compounds nitropyrene, *m*-dinitrobenzene and *p*-nitrobenzoic acid (*22*, *23*). More recenly, tetrachloro- and tetrafluorohydroquinone have been associated with a pro-redox cycling activity, assuming the corresponding benzoquinone metabolites to exert oxidative stress according to this mechanism (*27*).

In the present investigation, 19 nitroaromatic compounds including two phenol derivatives and the two herbicides trifluralin and dinitramine are studied with respect to their algal toxicity as determined in the 24 h reproduction test of the unicellular green algae *Scenedesmus vacuolatus* (formerly called *Chlorella fusca*). With the experimental results, quantitative structure-activity analyses are performed in order to elucidate underlying modes of toxic action.

To this end, hydrophobicity in terms of log K_{ow} and various quantum chemically calculated electronic parameters including E_{LUMO} and the net atomic charge at the nitro nitrogen, $q_{nitro\text{-}N}$, are used to describe the hydrophobic interaction potential and chemical reactivity profile of the molecular structures. For the two phenols, dissociation under the pH of the biotest medium is accounted for by correcting hydrophobicity for ionization (*21*). Moreover, the disposition for oxidative stress is studied by comparing calculated energy levels of the singly occupied molecular orbital, E_{SOMO}, of the radical anions generated by one-electon reduction with corresponding values of known redox cyclers. The discussion includes a detailed analysis of alternative toxic mechanisms and associated metabolic pathways.

Table 1. Nitroaromatic compounds with experimental and calculated data.

	Compound	Experimental[a]			Calculated[c]		
		log EC_{50} [mol/L]	Hill or Weibull fit[b]	log K_{ow}	E_{LUMO} [eV]	$q_{nitro\text{-}N}$ [a.u.]	E_{SOMO} [eV]
1	1-NO_2 (nitrobenzene)	-3.58	H	1.85	-1.068	0.567	1.145
2	2-Cl-1-NO_2	-3.82	H	2.24	-1.077	0.567	0.875
3	3-Cl-1-NO_2	-5.11	W	2.46	-1.285	0.568	0.817
4	4-Cl-1-NO_2	-4.66	W	2.39	-1.344	0.569	0.779
5	3-NH_2-1-NO_2	-3.56	H	1.37	-0.950	0.565	1.114
6	4-NH_2-1-NO_2	-3.48	H	1.39	-0.705	0.579	1.114
7	4-Me-1-NO_2	-3.90	W	2.37	-1.045	0.569	1.061
8	3,4-di-Cl-1-NO_2	-5.78	W	3.12	-1.524	0.570	0.508
9	2,3-di-NH_2-1-NO_2	-3.62	W	1.27	-0.842	0.577	0.924
10	2-NH_2-5-Cl-1-NO_2	-4.26	W	2.72	-1.003	0.583	0.638
11	4-Cl-3-Me-1-NO_2	-4.89	W	2.90	-1.283	0.569	0.774
12	4-NH_2-3-Me-1-NO_2	-4.04	H	1.83	-0.757	0.577	1.108
13	2-NPr_2-5-CF_3-1,3-NO_2 (trifluralin)	-7.14	H	5.34	-1.784	0.573	-0.272
14	2-NEt_2-5-CF_3-1,3-NO_2 (dinitramine)	-7.34	H	4.3	-1.696	0.599	-0.091
15	3-CF_3-4-NO_2-1-OH (TFM)	-4.78	W	2.77)[d]	-1.587	0.572	0.334
16	2-OH-1,3,5-NO_2	-2.96	W	0.89)[d]	-2.534	0.585	-0.613
17	2,4,5-tri-Cl-1-NO_2	-5.71	H	3.48	-1.534	0.570	0.275
18	1,3-di-NO_2	-5.60	H	1.49	-1.912	0.571	0.234
19	4-Cl-1,3-di-NO_2	-5.53	H	2.17	-2.062	0.573	-0.028

[a] EC_{50} data refer to reproduction inhibition within 24 h exposure of the algae *Scenedesmus vacuolatus*. Experimental log K_{ow} data are taken from (*29*), and CLOGP (*30*) was used to calculate log K_{ow} for compounds nr. 10, 11, 15.
[b] Dependent on the trend of the experimental data, the associated dose-response curve was derived using a 2-parameter Hill function or a 2-parameter Weibull function.
[c] E_{LUMO}, $q_{nitro\text{-}N}$ (net atomic charge of nitrogen in nitro group, taking maximum value in case of multiple nitro substitution) and E_{SOMO} were calculated with AM1 (*32*) including geometry optimization. E_{SOMO} refers to the radical anion generated by one-electron reduction of the nitroaromatic compound.
[d] With pK_a values of 6.13 for TFM and 0.33 for picric acid (*34*), correction of log K_{ow} for dissociation at pH 6.7 of the biotest medium due leads to log $D_{ow}{}^{u}$ and log D_{ow} of 2.10 and 2.11 (TFM) as well as of -5.48 and -1.11 (picric acid), respectively (cf. Eqs. 1 and 2).

Materials and Methods

Proliferation toxicity towards algae of 19 nitroaromatic compounds was determined using the 24-h reproduction assay of *Scenedesmus vacuolatus* (*28*) as described below. The chemicals were used as purchased from four different companies in Germany (Merck, Darmstadt: 1, 3-8, 10, 12, 16, 18, 19; Aldrich, Steinheim: 2, 15; Fluka, Neu-Ulm: 9, 11; Riedel, Seelze: 13, 14, 17), with purities ranging from >97% to >99%. The compound set is listed in Table 1 together with toxicity data and molecular descriptors.

Experimental procedure

Synchronous cultures of the unicellular green algae *Scenedesmus vacuolatus* (formerly called *Chlorella fusca*) were used, measuring inhibition of reproduction during one generation cycle of 24 h as toxicity endpoint (*28*). Photoautotrophic growth of the cells was performed at 28±0.5 °C in gas-tight test vessels with a standard cell density of $1x10^5$/mL. Synchronization of the cells was imposed prior to testing by a light-dark change of 14:10 hours and a periodic dilution to the standard cell density. After 24 hours, the cell number was analyzed, and comparison of cells exposed to a toxicant with control cultures yielded a quantification of the reduction in cell reproduction. The latter was expressed in terms of statistically calculated EC_{50} values, denoting the compound concentration that leads to a reduction of the cell reproduction by 50%. The pH of the biotest medium was 6.7. For more details of the biotest system, the reader is

referred to (*28*).

Chemical analysis of the nitroaromatics was performed by applying RP-HPLC, using a mobile phase of methanol/water (60:40 v/v) with two exceptions: For 2,4,6-trinitrophenol (picric acid), acetonitril/water (30:70 v/v) plus 0.1% phosphoric acid was used, and methanol/water (70:30 v/v) was employed for trifuralin. Quantification with a diode array detector was possible down to 10 μmol/L, which was sufficient for all biotest experiments.

Molecular descriptors

The compounds were characterized by parameters quantifying their hydrophobicity and various aspects of molecular reactivity. Hydrophobicity was expressed in terms of octanol/water partition coefficients in logarithmic form, log K_{ow}, which were taken from literature (*29*) or calculated using CLOGP (*30*) as specified in Table 1.

Initial geometries of the molecular structures were generated using the SYBYL software (*31*), and subsequently the semiempirical quantum chemical scheme AM1 (*32*) as implemented in MOPAC93 (*33*) was used to calculate the following 16 electronic parameters characerizing various aspects of donor and acceptor reactivities (*21*) from geometry-optimized structures of the molecules: Energy of the lowest unoccpied molecular orbital E_{LUMO}, energy of the highest occupied molecular orbital E_{HOMO}, energy of the singly occupied molecular orbital of the respective radical anion generated by 1-electron reduction E_{SOMO}, electronegativity *EN*, hardness χ, maximum net atomic charge at nitro nitrogen, nitro oxygen and of nitro group, $q_{nitro\text{-}N}$, $q_{nitro\text{-}O}$ and q_{NO2}, maximum nucleophilic (acceptor) and electrophilic (donor) delocalizabilities, D^N and D^E, of aromatic carbon as well as of nitro nitrogen, nitro oxygen and of the nitro group (see (*21*) for details of the calculation procedure).

For TFM (3-trifluoromethyl-4-nitrophenol) and picric acid (2,4,6-trinitrophenol) with pK_a values of 6.13 and 0.33, respectively (*34*), distribution coefficients at the biotest bioassay medium pH of 6.7 were derived from K_{ow} and the unionized and ionized compound fractions in water, f_u and $f_i = 1 - f_u$. While $D_{ow}{}^u$ quantifies the partitioning into octanol of the undissociated species available at the given pH D_{ow} accounts also for contributions from ion partitioning and ion-pair distribution,

$$D_{ow} = f_u K_{ow} + (1 - f_u)\frac{K_{ow}}{1 + 10^{pH - pK_a}} = D_{ow}^u + D_{ow}^i \qquad (1)$$

where the latter are denoted by K_i and K_{ip},

(2)

respectively (*21*). Since for several hydrophobic organic acids, $K_i + K_{ip}$ was below K_{ow} by a factor of at least 100 (*35*), $K_i + K_{ip} = K_{ow}/100$ was set as rough upper-limit estimate of the unknown contribution to D_{ow} from the hydrophobic anions. It follows that for a given concentration in water, c_w, of an organic acid, the respective concentration in octanol, c_o, is driven by both K_{ow} and pK_a, and can be written as

$$c_o = D_{ow} \bullet c_w = c_o^u + c_o^i \approx f_u K_{ow} c_w + (1 - f_u)\frac{K_{ow}}{100} c_w \qquad (3)$$

with $c_o{}^u$ and $c_o{}^i$ referring to the unionized and ionized species. The associated compound fractions in octanol, f_u^{oct} and f_i^{oct}, were then calculated according to

$$f_u^{oct} = \frac{c_o^u}{c_o} = \frac{100 f_u}{99 f_u + 1} \qquad (4)$$

and $f_i^{oct} = 1 - f_u^{oct}$. Accordingly, the reactivity descriptors *X* of TFM and picric acid were calculated for both the neutral and anionic form, HA and A^-, with

$$X\,(AH, A^-) = f_u^{oct} X\,(AH) + f_i^{oct} X\,(A^-) \qquad (5)$$

representing a rough estimate of their effective values under the pH of the bioassay medium when considering contributions from both molecular species.

Results

Algae toxicity. The 24-h reproduction toxicity of the 19 nitroaromatic compounds towards the algae *Scenedesmus vacuolatus* is expressed as decadic logarithm of the respective EC_{50} in mol/L. The results are listed in Table 1 together with (experimental or calculated) log K_{ow} data and the quantum chemically calculated electronic parameters E_{LUMO}, $q_{nitro\text{-}N}$ and E_{SOMO}.

As can be seen from Table 1, algal toxicity spans a range of 4.4 orders of magnitude. The lowest toxicity is exerted by picric acid (2,4,6-trinitrophenol) with an EC_{50} of 1.10×10^{-3} mol/L, and the highest toxicities are observed for dinitramine and trifuralin with EC_{50} values of 4.59×10^{-8} mol/L and 7.28×10^{-8} mol/L, respectively. Note further that the overall variation of EC_{50} is very similar to the one of K_{ow}, which latter covers 4.5 orders of magnitude.

Figure 1 shows the sigmoid dose-response curves, which were fitted using Hill or Weibull functions depending on the shape of the experimental trend as specified in Table 1. Interestingly, 2,3-diaminonitrobenzene (9) and 4-chloro-1,3-dinitrobenzene (19) have significantly smaller slopes than all other compounds. This is further illustrated by evaluating the ratio EC80/EC20 derived from the fitted functions, which is below 5 for all compounds except 9 and 19, where EC80/EC20 amounts to approximately 6 and 23, respectively.

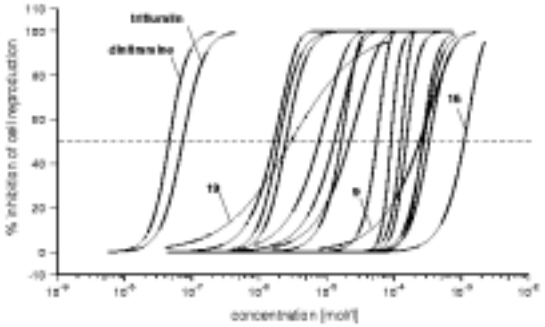


Figure 1. Concentration-effect curves of all 19 nitroaromatics (cf. Table 1) in the 24 h reproduction toxicity test with the algae *S. vacuolatus*.

Principal component analysis. A means to unravel common features behind the variation of individual compound properties is given by the multivariate statistical method principal component analysis (*36*). In the present case, the quantum chemical AM1 method has been used to characterize various aspects of the electronic polarizability, electron donor and acceptor reactivity as well as the reactivity for oxidation and reduction, making up a total of 16 parameters as described above. Inclusion of log K_{ow} and all 16 reactivity parameters leads to Figure 2, where the scores (representing the coordinates of the 19 compounds with respect to the derived principal components) reveal some interesting patterns.

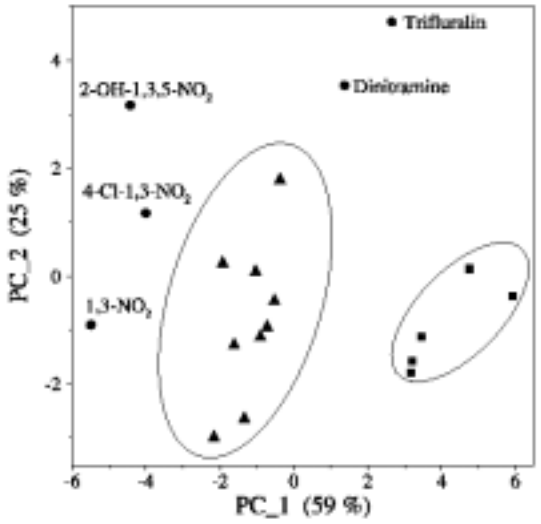


Figure 2. Compound scores resulting from a principal component analysis including log K_{ow} and all 16 reactivity descriptors of the 19 nitroaromatics (cf. Materials and Methods and Table 1), covering 84% of the total variance with the first two principal components. The five mononitro amines are denoted with squares, while triangles denote the nine remaining mononitro derivatives. The location of the two clusters is indicated by closed lines around the respective scores.

As can be seen from the figure, one cluster of compounds with correspondingly similar property profiles is given by the five mononitroanilines (5, 6, 9, 10, and 12), and another cluster includes the remaining nine mononitro

derivatives (1-4, 7, 8, 11, 15, and 17). The four dinitro compounds as well as picric acid as only trinitro derivative are located outside these clusters, with dinitramine and trifluralin forming a further subset. It would be tempting to explain the latter by the much higher hydrophobicity of these two compounds as compared to the other chemicals, but omission of log K_{ow} in a separate principal component analysis leads to a very similar picture (results not given), showing that these more complex structures differ also in their intrinsic reactivity pattern from all other nitroaromatic compounds under analysis.

According to Figure 2, the numerical information encoded in the total set of 17 molecular descriptors is able to differentiate between congeneric subsets of the compounds as mentioned above. However, the calculated property profile does not yield a specific commonality between the only two phenols TFM and picric acid, the former of which is included in the mononitrobenzene cluster.

Linear regression on hydrophobicity. The plot of log EC_{50} vs. log K_{ow} in Figure 3 shows that there exists a linear relationship between algae toxicity and hydrophobicity except for the three dinitro derivatives dinitramine, 1,3-dinitrobenzene and 4-chloro-1,3-dinitrobenzene:

$$\log EC_{50}[\text{mol/L}] = -0.96\ (\pm 0.09) \bullet \log K_{ow} - 2.15\ (\pm 0.23) \quad (6)$$
$$r^2_{adj} = 0.89,\ r^2 = 0.90,\ SE = 0.36,\ F_{1,14} = 120,\ n = 16$$

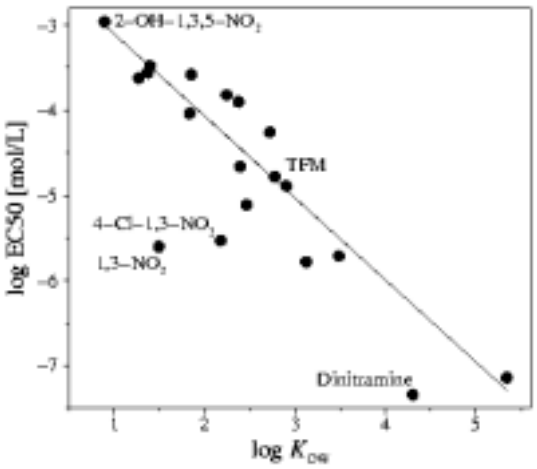
Inspection of the data distribution reveals that the three outlying dinitrobenzenes possess excess toxicities EC_{50}(pred.)/EC_{50}(exp.) of one to two orders of magnitude when applying Eq. 6 for predicting their log EC_{50} values. By contrast, the algae toxicities of the two additional compounds with more than one nitro group, trifluralin and picric acid, do not deviate substantially from the values predicted by Eq. 6, and TFM as second acidic compound also fits well to the hydrophobicity-dependent regression line when using the log K_{ow} value of the undissociated structure.

Figure 3 suggests further that the overall statistics of Eq. 6 depend significantly on the entry of trifluralin with an EC_{50} far below the remainder of the subset. Elimination of this compound as well as of picric acid with the lowest toxicity reduces the K_{ow} variation from 4.5 to 2.2 log units

$$\log EC_{50}[\text{mol/L}] = -1.02\ (\pm 0.16) \bullet \log K_{ow} - 2.02\ (\pm 0.\ \ ) \quad (7)$$
$$r^2_{adj} = 0.76,\ r^2 = 0.78,\ SE = 0.39,\ F_{1,12} = 42,\ n = 14$$

and leads to

as regression equation for the subset of 14 mononitro aromatics. While the statistics are significantly inferior, both regression coefficient and intercept are close to the corresponding values



of Eq. 6.

Figure 3. Plot of the experimental log EC_{50} vs log K_{ow} for the 19 nitroaromatics of Table 1. The regression line according to eq 6 refers to the subset of 16 compounds excluding dinitramine, 1,3-dinitrobenzene, and 4-chloro-1,3-dinitrobenzene.

In aqueous solution, the degree of deprotonation of TFM and picric acid depends on their pK_a (6.13 for TFM and 0.33 for picric acid) and the pH (6.7) of the medium. For the anilines, protonation is not relevant at the given pH, because the (calculated) pK_a values of all conjugate acids are below 2.5. For TFM and picric acid, correction of the octanol/water partition coefficient for ionization according to Eq. 1 leads to log D_{ow}^{u} values of the undissociated compound fraction of 2.10 and -5.48, respectively. Introduction of these values in Eq. 6 results in substantial underestimations of the algae toxicity by 0.61

(TFM) and 6.07 (picric acid) log units. Interestingly, log D_{ow} that accounts for both the neutral and anionic form yields significantly inferior regression statistics for the 16 compounds (r^2_{adj} = 0.77, SE = 0.52) as well as for the subset of 14 mononitro derivatives (r^2_{adj} = 0.72, SE = 0.42).

It follows that for the present data set, log K_{ow} is apparently preferred to both log D_{ow}^{u} and log D_{ow} for quantifying hydrophobicity in one-parameter QSARs, which implies that in this case the deprotonation of ionogenic nitroaromatics apparently should be ignored.

Multilinear regression including hydrophobicity and molecular reactivity. According to the fact that an important biotransformation route of nitroaromatics is their step-wise reduction to aromatic amines, the susceptibility for reduction in terms of E_{LUMO} (energy of the lowest unoccupied molecular orbital) is of interest as potentially relevant reactivity parameter. Bilinear regression of log EC_{50} on both log K_{ow} (ignoring ionization) and E_{LUMO} yields

$$\log EC_{50}[\text{mol/L}] = -0.61\ (\pm 0.11)\log K_{ow} + 1.564\ (\pm 0.298)$$
$$E_{LUMO} - 1.24\ (\pm 0.36)$$
$$r^2_{adj} = 0.87,\ r^2 = 0.89,\ SE = 0.42,\ F_{2,15} = 59,\ n = 18 \qquad (8)$$

upon omission of picric acid, the only compound that is deprotonated to (even much) more than 99.9% under the pH of the biotest medium. The signs of both regression coefficients are in accord with expectation: Toxicity increases with decreasing log EC_{50}, which in turn decreases with increasing log K_{ow} and greater negative E_{LUMO} (cf. Table 1).

As compared to Eq. 6, the two-variable relationship of Eq. 8 yields somewhat inferior statistics, but includes all dinitro derivatives, thus accounting for the increased reduction potential of these compounds. Interestingly, the regression coefficient of log K_{ow} in Eq. 8 is significantly smaller in absolute size than in Eq. 6, showing that the latter is partly containing E_{LUMO}-related information due to a moderate intercorrelation between log K_{ow} and E_{LUMO} (r^2 = 0.27 for the 18-compound subset).

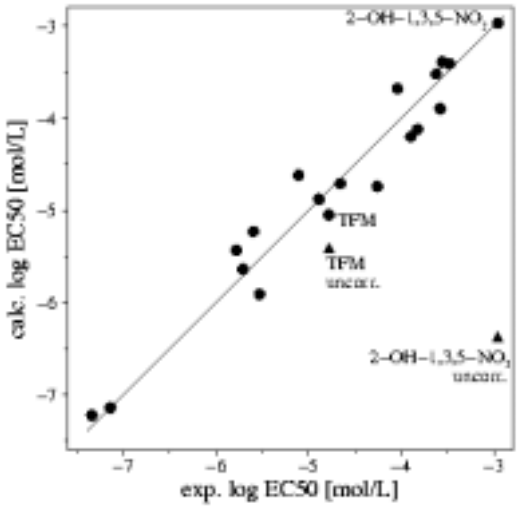
With this subset of 18 compounds, E_{LUMO} yields the best two-variable relationship together with log K_{ow} among all 16 electronic parameters. Moreover, separate linear regressions of log EC_{50} on log K_{ow} alone and on E_{LUMO} alone give significantly inferior statistics with r^2_{adj} values of 0.66 and 0.65, respectively, and all other electronic parameters (except E_{SOMO}, s.b.) are much inferior to E_{LUMO} as single descriptor for log EC_{50}.

Among the remaining electronic parameters, $q_{nitro\text{-}N}$ is the only significant third descriptor in combination with log K_{ow} and E_{LUMO}, providing a still substantial improvement of the quality of fit:

$$\log EC_{50}[\text{mol/L}] = -0.54\ (\pm 0.10)\log K_{ow} + 1.674\ (\pm 0.261)$$
$$E_{LUMO} - 35.530\ (\pm 14.597)\ q_{nitro\text{-}N} - 19.08\ (\pm 8.33)$$
$$r^2_{adj} = 0.91,\ r^2 = 0.92,\ SE = 0.36,\ F_{3,14} = 56,\ n = 18 \qquad (9)$$

The intercorrelations of $q_{nitro\text{-}N}$ with E_{LUMO} (r^2 = 0.003) and log K_{ow} (r^2 = 0.10) are quite small, and $q_{nitro\text{-}N}$ alone can explain only 16% of the variation of log EC_{50}. The negative sign of the regression coefficient of $q_{nitro\text{-}N}$ indicates that toxicity increases with a greater positive charge at the nitro group nitrogen.

Figure 4. Calculated vs experimental log EC_{50}



using the three-variable regression model of eq 11. For TFM and picric acid (2,4,6-trinitrophenol), the triangles indicate their predicted log EC_{50} when using log K_{ow} instead of D_{ow}^{u}.

Although Eqs. 6-9 provide satifactory statistics, the treatment of the nitroaromatic phenols is not straightforward. While both TFM and picric

acid fit well to Eq. 6, the latter compound is not covered by Eqs. 8 and 9 that account explicitely for some contribution from molecular reactivity. Correction of log K_{ow} for dissocation in terms of log D_{ow}^{u} as outlined above (Eq. 1) now leads to

$$\log EC_{50}[\text{mol/L}] = -0.53\ (\pm 0.05) \log D_{ow}^{u} + 1.776\ (\pm 0.201)$$
$$E_{LUMO}\ -1.15\ (\pm 0.33)$$
$$r^2_{adj} = 0.90,\ r^2 = 0.91,\ SE = 0.39,\ F_{2,16} = 82,\ n = 19 \qquad (10)$$

which includes all 19 compounds. The corresponding 3-variable equation with $q_{nitro\text{-}N}$ as additional descriptor reads

$$\log EC_{50}[\text{mol/L}] = -0.55\ (\pm 0.04) \log D_{ow}^{u} + 1.690\ (\pm 0.170)$$
$$E_{LUMO}\ -34.335\ (\pm 11.608)\ q_{nitro\text{-}N}\ -18.43\ (\pm 6.63)$$
$$r^2_{adj} = 0.93,\ r^2 = 0.95,\ SE = 0.32,\ F_{3,15} = 84,\ n = 19 \qquad (11)$$

with the data distribution of predicted vs. experimental log EC_{50} shown in Figure 4. Both Eqs. 10 and 11 have significantly improved statistics as compared to Eqs. 8 and 9, also as regards the standard errors of the regression coefficients. Note further that the regression coefficient of log D_{ow}^{u} in Eqs. 10 and 11 is similar to the one of log K_{ow} in Eqs. 8 and 9, and consequently smaller in absolute size than the log K_{ow} coefficient in Eqs. 6 and 7. It reflects a significant influence of E_{LUMO} as second descriptor, which in this case, however, is not obvious from its only small intercorrelation with log D_{ow}^{u} (r^2 = 0.10); the corresponding intercorrelations of $q_{nitro\text{-}N}$ are still smaller (r^2 = (-)0.02 with log D_{ow}^{u}, and r^2 = 0.06 with E_{LUMO}).

From a mechanistic viewpoint, the use of log D_{ow}^{u} implies that the ionized compound portion does not contribute to toxicity, which contrasts with evidence from other data sets, e.g. an apparent concentration addition of the acidic and anionic form of chlorophenols with respect to their acute fish toxicity (*21*). As outlined above, the anion and ion-pair partitioning of deprotonated organic acids into octanol can be roughly estimated by an upper limit of K_{ow}/100 (with K_{ow} referring to the neutral species), resulting in log D_{ow} values of 2.11 for TFM and -1.11 for picric acid (Eq. 2). Following Eq. 4, the respective unionized compound fraction in octanol is 96.4% for TFM and only $4.3*10^{-3}$% for picric acid. It follows that for the latter compound, the anionic form would actually be the dominant xenobiotic species in the algae tissue. Replacement of log D_{ow}^{u} by log D_{ow} in Eq. 10 leads to inferior statistics,

$$\log EC_{50}[\text{mol/L}] = -0.79\ (\pm 0.08) \log D_{ow} + 1.192\ (\pm 0.214)$$
$$E_{LUMO}\ -1.28\ (\pm 0.37)$$
$$r^2_{adj} = 0.87,\ r^2 = 0.89,\ SE = 0.43,\ F_{2,16} = 64,\ n = 19 \qquad (12)$$

and here $q_{nitro\text{-}N}$ is not a significant third descriptor. Note, however, that the log D_{ow} coefficient is in between the corresponding regression coefficients in Eqs. 8-11 (log D_{ow}^{u}, log K_{ow}) and Eqs. 6 and 7 (log K_{ow}), and that the E_{LUMO} coefficient is significantly smaller than in the other regression equations.

While log D_{ow} in Eq. 12 accounts for both the unionized and ionized compound fraction, E_{LUMO} refers only to the neutral species. For the anions of TFM and picric acid, E_{LUMO} is higher by about 4.5 eV (3.132 eV vs. -1.587 eV and 1.892 eV vs. -2.534 eV, respectively), indicating that these deprotonated species have no tendency for electronic reduction. Interestingly, $q_{nitro\text{-}N}$ is even more positive for the anions than for the neutral species (0.594 a.u. vs. 0.572 a.u. for TFM, and 0.595 a.u. vs. 0.585 a.u. for picric acid) despite the overall negative charge. Following Eq. 5, effective E_{LUMO} and $q_{nitro\text{-}N}$ values can be calculated that reflect the relative portions of neutral and anionic species present in octanol. The resultant values are -1.417 eV and 0.573 a.u. for TFM, and 1.892 eV and 0.595 a.u. for picric acid.

Introduction of these effective reactivity parameters for TFM and picric acid in Eq. 11, however, eliminates E_{LUMO} as significant descriptor, thus leaving log D_{ow} as only descriptor that does not provide an adequate fit of the data (r^2_{adj} = 0.65 and SE = 0.72). The corresponding modification of Eq. 12 results in rather low overall statistics (r^2_{adj} = 0.75, SE = 0.61) that cannot compete with any of the other regression equations including hydrophobicity and molecular reactivity. It follows that for QSARs derived from the present data set with (however only) two ionogenic compounds, the use of log D_{ow}^{u} and reactivity

parameters of the undissociated molecules is clearly preferred over parameters that account explicitely for the presence of the anionic species.

Comparison with redox cyclers. As outlined above, nitroaromatic compounds belong to the group of compounds that may exert oxidative stress by acting as redox cyclers. For this mode of action, the nitroaromatic radical anion formed by one-electron reduction is oxidized back to the parent compound while forming superoxide (O_2^-), which then leads to the generation of hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) as highly reactive oxidants. Apparently, redox cycling potency thus competes with the ability of further reduction.

From these considerations it follows that the energy of the singly occupied molecular orbital, E_{SOMO}, of the radical anion may be a suitable parameter for differentiating between redox-cycling compounds and those that are preferentially being reduced further to metabolites with closed-shell electronic structures (like nitroso derivatives and hydroxylamines in the case of nitroaromatic chemicals). In Table 2, E_{SOMO} values of the radical anions of eight known redox cyclers (compounds 20-27) taken from literature (*22-25*, *37-38*) and of two 2,3,5,6-tetrahalogenated benzoquinones (28-29) that were also assigned to exert redox-cycling toxicity (*27*) are listed together with the E_{LUMO} values of the parent compounds.

Table 2. Redox cyclers and oxidizing agents.[a]

	Compound	E_{LUMO} [eV]	E_{SOMO} [eV]
20	nitrofurantoin	-1.613	-0.240
21	nifurtimox	-1.490	-0.076
22	menadione	-1.493	0.401
23	naphthalene-1,2-dione	-1.518	0.410
24	phenanthrene-9,10-dione	-1.319	0.272
25	7,12-dimethyl-benz[a]anthracene-3,4-dione	-1.208	0.173
26	p-nitrobenzoic acid	-1.730	0.124
27	2,3,5,6-tetramethylbenzoquinone	-1.505	0.501
28	2,3,5,6-tetrafluorobenzoquinone	-2.735	-0.469
29	2,3,5,6-tetrachlorobenzoquinone	-2.427	-0.475

[a] Compounds #20, #22 taken from (*22-24*), #21 from (*37*), #23-25 from (*38*), #26 from (*22*), #27 from (*25*), and #28-29 from (*27*). Molecular orbital energies E_{LUMO} and E_{SOMO} (the latter referring to the radical anion generated by one-electron reduction) are calculated with AM1 (*32*) from geometry-optimized molecular structures.

For the two nitrofurans, four polycyclic aromatic hydrocarbon (PAH) quinones, *p*-nitrobenzoic acid and tetramethylbenzoquinone with demonstrated redox cycling activity, E_{LUMO} is below -1.200 eV, and E_{SOMO} ranges from -0.240 eV (nitrofurantoin) to 0.501 eV (2,3,5,6-tetramethylbenzoquinone). These data suggest a tentative E_{SOMO} window as molecular disposition for efficient redox cycling of about -0.30 to 0.55 eV, provided that E_{LUMO} is sufficiently low to allow the one-electron reduction step of the parent compound.

According to the tentatively derived E_{SOMO} criterion, the radical anions of tetrafluoro- and tetrachlorobenzoquinone are likely to be preferentially reduced further instead of being oxidized back to the parent compound. With the same reasoning, oxidative stress via redox cycling would be expected to form a possible toxicity mechanism for the four dinitro derivatives (13,,14, 18,19) as well as for TFM (15), 3,4-dichlorobenzene (8) and 2,4,5-trichlorobenzene (17).

Discussion

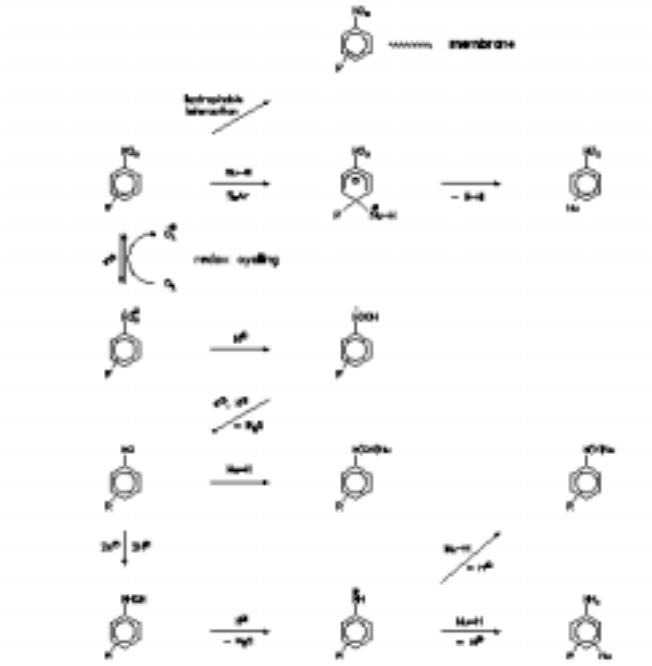
The toxicity of nitroaromatic compounds appears to involve several modes of action as outlined in Scheme 1. Besides impairments of membrane constituents through hydrophobic interaction, there are several distinct metabolic pathways that may vary in their actual relevance depending on the biological species as well as on the intrinsic reactivity pattern of the compounds.

For nitrobenzene derivatives with a sufficient electron deficiency at the aromatic ring due to strong electron-accepting substituents, an addition-elimination reaction with endogeneous nucleophiles according to the S_NAr mechanism may take place, which has been discussed to form the major route for skin sensitizing compounds (*39*, *40*). Alternatively, step-wise reduction may lead to nitroso and hydroxylamine metabolites (*7*, *9*, *15*), which both possess enhanced electrophilicity to interact with electron-rich sites of macromolecules.

Moreover, the radical anion formed by one-electron reduction may be oxidized back to the parent compound, forming O_2^- that in turn generates further oxidant agents such as H_2O_2 and OH^-, thus leading to oxidative stress and cytotoxic effects (*22-26*). Whether or not an efficient oxidation/reduction cycle of the nitroaromatic

compound occurs, depends on the balance between the oxidative and reductive pathway of the initially formed radical anion, and probably also on further aspects like molecular hydrophobicity and physiological properties of the organism. Clearly, the variety of metabolic routes and bioactive agents formed from nitroaromatic compounds makes it difficult to decide which of the different modes of action will be of primary importance for a given test organism and endpoint.

Scheme 1. Toxic Modes of Action of Nitroaromatics, Including Accociated Metabolic Pathways[a]



[a] Nu denotes endogenous nucleophiles like nucleic acids and proteins.

Considering our test set of nitrobenzene and 18 derivatives, the QSAR of Eq. 6 as shown in Figure 3 suggests that for the mononitro compounds a narcotic-type syndrome can sufficiently explain the toxic action towards algae. The negative sign of the regression coefficient (-0.96) is in line with expectation: EC_{50} increases with decreasing K_{ow}; thus increasing hydrophobicity increases the algal toxicity of the compounds.

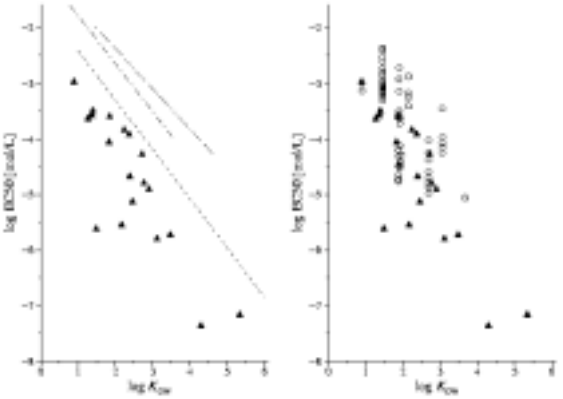


Figure 5. Log EC_{50} with different algal species vs log K_{ow} for the following data sets. (A) Experimental values for 19 nitroaromatics (*S. vacuolatus*, 24 h exposure, ▲) and three linear regression lines for nonpolar narcotics [*S. Subspicatus* (18), 48 h exposure, eight compounds, dashed line; *Se. capricornutum* (19), 96 h exposure, five compounds, dotted line; *Sk. costatum* (41), 96 h exposure, nine compounds, dashed and dotted linear regression]. (B) Experimental values for 19 nitroaromatics (*S. vacuolatus*, 24 h exposure, ▲) and 76 data points for polar narcotics taken from ref 43.

The apparently narcotic mode of action is supported further by comparison with literature data on the algal toxicity of nonpolar and polar narcotics as shown in Figure 5. As can be seen from the left part of the figure, the toxicity of the presently analyzed 19 nitroaromatics with respect to *Scenedesmus vacuolatus* lies about one order of magnitude above the QSAR regression lines of three sets of nonpolar narcotics tested with *Scenedesmus subspicatus* (*18*), *Scenedesmus capricornutum* (*19*) and *Scenedesmus costatum* (*41*).

Moreover, the right part of Figure 5 shows no significant difference between the EC_{50} range of the present test set and the algal toxicity range of polar narcotics selected according to the criteria for this syndrome (*42*) from the AQUIRE database (*43*). These findings suggest a classification of mononitroaromatic compounds as polar narcotics, although the actual mechanism behind this syndrome is still a matter of discussion (*21*).

Inclusion of E_{LUMO} to model the susceptibility for one-electron reduction is

straightforward, leading to Eq. 8 that now includes all dinitro derivatives. However, Eq. 8 is not able to discriminate between different pro-electrophilic mechanisms (electrophilic attack of nitroso or hydroxylamine metabolites at endogeneous macromolecules) and oxidative stress through redox cycling, all of which require a sufficiently strong electron affinity to become initiated by the reduction as common first biotransformation step.

The fact that trifluralin as dinitro derivative fits well to Eq. 6 despite its relatively low E_{LUMO} value may be explained by its pronounced hydrophobicity with a log K_{ow} of 5.34: Although biotransformation according to the routes outlined in Scheme 1 is likely to take place for this compound, the resulting toxicity enhancement may be small as compared to the already high toxicity associated with the high hydrophobicity. Note a corresponding situation with the acute fish toxicity of pentachlorophenol, which is known to exert oxidative uncoupling (*44*) but nonetheless does not deviate significantly from the QSAR regression line predicted from log K_{ow} alone (*45*).

A less straightforward aspect is the finding that picric acid (2,4,6-trinitrophenol) as almost completely dissociated compound under the biotest conditions fits well to Eq. 6, but not to Eq. 8. The latter is particularly remarkable in view of the fact that among the 19 compounds under analysis, picric acid is the least toxic compound despite a very strong electron affinity as characterized by its E_{LUMO} value of -2.534 eV, which is below the E_{LUMO} values of all other test compounds. Note further that 1,3-dinitrobenzene is more hydrophobic than picric acid by only 0.6 log K_{ow} units, but more toxic by 2.6 orders of magnitude, with a less negative E_{LUMO} value of -1.912 eV (cf. Table 1). The latter illustrates further that either log K_{ow} (uncorrected for ionization) or E_{LUMO} (referring to the parent compound) or both are inappropriate as molecular desciptors for picric acid in the context of its only moderate algal toxicity.

When considering both the correction of hydrophobicity for ionization in terms of log D_{ow}^{u} and the molecular reactivity, the extreme deviation of the log EC_{50} of picric acid from nitroaromatic baseline toxicity as defined by Eq. 6 when using log D_{ow}^{u} alone makes sense: The already small log K_{ow} of 0.89 is in fact much too high and yields a fit of picric acid to baseline toxicty by coincidence, masking the bioreactive contribution to toxicity. The log D_{ow}^{u} value of -5.48 brings picric acid on the correct hydrophobicity scale (while neglecting contributions from ion partitioning and ion-pair distribution (*21*)), and now inclusion of E_{LUMO} according to Eq. 10 is suitable to explain the actual toxicity of picric acid as compared to non-dissociating nitroaromatic compounds.

In a similar but much less pronounced way due to the much smaller difference between pK_a and pH, the exellent fit of TFM to Eq. 6 also results from the fact, that the too high (uncorrected) log K_{ow} of the undissociated species accounts indirectly for the bioreactive contribution to toxicity. As can be seen from inspection of Figure 4, the fit for both TFM and picric acid improves significantly when using log D_{ow}^{u} in combination with descriptors for the electron acceptor capability of the compounds.

Interestingly, inclusion of the maximum net atomic charge at the nitrogen of the nitro group, $q_{nitro\text{-}N}$, as third descriptor yields a further significant improvement of the regression statistics. Although the variation in the net atomic charge of the nitro nitrogen is only small (0.034 a.u., see Table 1), its contribution to explaining part of the toxicity makes sense, as the nitro nitrogen is likely to be the site of attack of the additional negative charge upon the one-electron reduction of the molecule. Note further that there is almost no intercorrelation of $q_{nitro\text{-}N}$ with E_{LUMO} (r^2=(-)0.06) and with log D_{ow}^{u} (r^2=(-)0.02) as well as with log K_{ow} (r^2=0.03).

On the one hand, the role of $q_{nitro\text{-}N}$ in Eq. 11 could be to correct for some deficiency of E_{LUMO} in modelling the susceptibility for one-electron reduction, as E_{LUMO} is only approximately representing the electron affinity of molecular species (cf. (*21*) and textbooks on quantum chemistry). On the other hand, it may also be possible that $q_{nitro\text{-}N}$ as additional descriptor would in principle allow a discrimination between different pro-electrophilic mechanisms, which is an interesting route of further investigation.

As outlined elsewhere (*21*), the electronegativity *EN* characterizes the electron attraction tendency of molecules, and as such could

be used in principle as alternative to E_{LUMO}. For the present data set, however, the statistics are clearly in favour of E_{LUMO}, which is also more directly related to the one-electron reduction process (*21*) as the metabolic step that is apparently rate-determining for the bioreactive toxicity contribution of the compounds. Similarly, the combination of log D_{ow}^{u} with both *EN* and χ would make sense mechanistically also from the signs of the regression coefficients (toxicity increases with increasing electronegativity and decreasing hardness, results not shown), but Eq. 11 with log D_{ow}^{u}, E_{LUMO} and $q_{nitro\text{-}N}$ can also be interpreted in a sound way and yields clearly superior statistics.

From a mechanistic viewpoint, consideration of both the neutral and anionic form of ionogenic compounds would be warranted for discussing their toxic effects on a molecular level. This is particularly true for stronger organic acids like picric acid, where the concentration of the anion *in vivo* is most likely to be much higher than that of the neutral contaminant. Surprisingly, the present results are not in accord with this expectation, which is seen by the fact that neither log D_{ow} alone (with $K_i + K_{ip}$ estimated as $K_{ow}/100$) nor log D_{ow} in combination with molecular reactivity parameters can compete with log K_{ow} or log D_{ow}^{u} in corresponding QSARs. Similarly, the correction of E_{LUMO} and $q_{nitro\text{-}N}$ for ionization according to Eq. 5 (as a certainly quite simple model) yields significantly inferior statistics when combined with log D_{ow} as well as with log D_{ow}^{u} or log K_{ow} (the latter results not shown). Note, however, that there were only two acidic compounds on which log D_{ow} (Eq. 2) and the effective reactivity parameters (Eq. 6) could actually be applied, and so the approach outlined in Eqs. 2-6 may still prove useful with other data sets including substantially more ionogenic compounds.

Considering oxidative stress through redox cycling as potentially relevant mechanism of toxicity of nitroaromatic compounds besides polar narcosis and pro-electrophilicity, analysis of the radical anion E_{SOMO} values of known redox cyclers (Table 2) has lead to the identification of an E_{SOMO} window of -0.30 eV to 0.55 eV as a tentative criterion for the ability of molecular structures to undergo efficient reduction/oxidation cycling, provided their E_{LUMO} is sufficiently low to enable an efficient one-electron reduction of the parent compound. According to this hypothesis, all dinitro derivatives (13-14, 18-19) and TFM (15) as well as di- and trichlorobenzene (8, 17) are candidates for oxidative stress. This is not necessarily in conflict with the narcotic-type dependence of log EC_{50} on log D_{ow}, as for some compounds like 2,3-dichlorobenzene a hypothetic toxicity enhancement through oxidative stress may well be small as compared to the hydrophobicity-driven unspecific membrane irritation.

Due to the high intercorrelation between E_{LUMO} and E_{SOMO} (r^2=0.86), a statistically based discrimination between the individual influences of both properties on toxicity by means of multlinear regression is not feasible. This is demonstrated by the regression of log EC_{50} on both log D_{ow}^{u} and E_{SOMO}

$$\log EC_{50}[\text{mol/L}] = -0.47\ (\pm 0.04) \log D_{ow}^{u} + 1.577\ (\pm 0.179)\ E_{SOMO} - 4.64\ (\pm 0.15)$$
$$r^2_{adj} = 0.90,\ r^2 = 0.91,\ SE = 0.39,\ F_{2,16} = 80,\ n = 19 \qquad (13)$$

which results in statistics almost identical to the ones of Eq. 10, including a similar regression coefficient for log D_{ow}^{u} (and with a low intercorrelation between both parameters of r^2 = 0.03). In this case, however, $q_{nitro\text{-}N}$ is not significant as third descriptor, which may also have to do with the fact that E_{SOMO} itsself can be modelled well by a bilinear regression on E_{LUMO} and $q_{nitro\text{-}N}$, yielding r^2=0.92. For the time being, we suggest to use E_{LUMO} (perhaps together with $q_{nitro\text{-}N}$ as additional descriptor) in combination with log K_{ow} or log D_{ow}^{u} for ionogenic compounds to screen the algal toxicity of nitroaromatic compounds, and to employ E_{SOMO} in combination with E_{LUMO} for identifying potential redox cyclers.

Conclusions

The toxicity of nitroaromatic compounds is likely to result from the superposition of different modes of action. This can be traced back to the intrinsic reactivity of these chemicals for reductive biotransformation reactions, which depend on the metabolic capacity and physiological parameters of the organism and lead to different molecular species as toxic agents *in vivo*.

Due to the electrophilic nature of the parent compounds and their nitroso metabolites, one mechanistic route to exert specific toxicity is the attack at electron-rich sites of endogenous macromolecules, resulting in malfunctions of proteins including enzymes and nucleic acids. Another route is the further reduction to hydroxylamines with their own toxicity profile, which also requires a certain electron attraction capacity. Thus, formation of hydroxylamine metabolites competes with electrophilic conjugation reactions of the precursor compounds, the balance of which is governed by details of the electronic structure of the chemicals that are still to be elucidated.

Moreover, the radical anions generated by one-electron reduction may also undergo redox cycling with subsequent oxidative stress and cytotoxicity. The present results suggest that the susceptibility of nitroaromatics for this mechanism can be evaluated by the energy of the singly occupied molecular orbital of the radical anion, E_{SOMO}, which can be calculated by quantum chemical methods from the molecular structure.

Finally, the hydrophobicity of nitroaromatics may lead to unspecific membrane irritation as a further mode of action, which may mask contributions from more specific toxic activities in the case of highly hydrophobic compounds. While hydrophobicity can be quantified by the decadic logarithm of the octanol/water partition coefficient, log K_{ow}, the susceptibility for one-electron reduction as common first step of the different metabolic pathways can be characterized by the energy of the highest occupied molecular orbital, E_{LUMO}. However, E_{LUMO} alone does not allow to discriminate between the different biotransformation routes of the radical anions formed initially, which latter is another open question for future investigations.

References

(1) Hartter, D.R. (1985) The use and importance of nitroaromatic chemicals in the chemical industry. In *Toxicity of nitroaromatic compounds* (Rickert, D.E., Ed.) pp 1-13, Hemisphere, New York, USA.

(2) Pitts, J.N., Jr.,, Van Cauwenberghe, K.A., Grosjean, D., Schmid, J.P., Fitz, D.R., Belser, W.L., Jr., Knudson, G.B., and Hynds, P.M. (1978) Atmospheric reactions of polycyclic aromatic hydrocarbons: Facile formation of mutagenic nitro derivatives. *Science* **202**, 515-519.

(3) Handa, T., Yamauchi, T., Sawai, K., Yamamura, T., Koseki, Y., and Ishii, T. (1984) In situ emission levels of carcinogenic and mutagenic compounds from diesel and gasoline engine vehicles on an expressway. *Environ. Sci. Technol.* **18**, 895-902.

(4) Arey, J. (1998) Atmospheric reactions of PAHs including formation of nitroarenes. In *The Handbook of Environmental Chemistry, Vol. 3, Part I. PAHs and Related Compounds* (Neilson, A.H., Ed.) pp 347-385, Springer-Verlag, Berlin, Germany.

(5) Bushby, W.F., Jr., Smitz, H., Crespi, C.L., Penman, B.W., and Lafleur, A.L. (1997) Mutagenicity of the atmospheric transformation products 2-nitrofluoranthene and 2-nitrodibenzopyranone in Salmonella and human cell forward mutation assays. *Mutat. Res.* **389**, 261-270.

(6) Rippen, G., Zietz, E., Frank, R., Knacker, T., and Klöpffer, W. (1987) Do airborne nitrophenols contribute to forest decline? *Environ. Technol. Letters* **8**, 475-482.

(7) Bailey, H.C., and Spanggord, R.J. (1983) The relationship between the toxicity and structure of nitroaromatic chemicals. In *Aquatic Toxicology and Hazard Assessment, Sixth Symposium, ASTM STP 802* (Bishop, W.E., Cardwell, R.D., and Heidolph, B.B., Eds.) pp

98-107, American Society for Testing and Materials, Philadelphia, USA.

(8) Deneer, J.W., Sinnige, T.L., Seinen, W., and Hermens, J.L.M. (1987) Quantitative structure-activity relationships for the toxicity and bioaccumulation factor of nitrobenzene derivatives towards the guppy (*Poecilia reticulata*). *Aquat. Toxicol.* **10**, 115-129.

(9) Roberts, D.W. (1987) An analysis of published data on fish toxicity of nitrobenzenes and aniline derivatives. In *QSAR in Environmental Toxicology - II* (Kaiser, K.L.E., Ed.) pp 295-308, D. Reidel, Dordrecht, NL.

(10) Purdy, R. (1988) Quantitative structure relationships for predicting toxicity of nitrobenzenes, phenols, anilines, and alkylamines to fathead minnows. In *QSAR 88, Proceedings of the Third International Workshop on Quantitative Structure-Activity Relationships in Environmental Toxicology* (Turner, J.E., England, M.W., Schultz, T.W., and Kwaak, N.J., Eds.) pp 99-110, US Department of Energy, Oak Ridge (TN), USA.

(11) Lang, P., Ma, X., Lu, G., and Bian, Y. (1996) QSAR for the acute toxicity of nitroaromatics to the carp (Cyprinus caprio). *Chemosphere* **32**, 1547-1552.

(12) Zhao, Y.-H., Yuang, X., Ji, G.-D., and Sheng, L.X. (1997) Quantitative structure-activity relationships of nitroaromatic compounds to four aquatic species. *Chemosphere* **34**, 1837-1844.

(13) Deneer, J.W., van Leeuwen, C.J., Seinen, W., Maas-Diepeveen, J.L., and Hermens, J.L.M. (1989) A QSAR study of the toxicity of nitrobenzene derivatives towards *Daphnia magna*, *Chlorella pyrenoidosa* and *Photobacterium phosphoreum*. *Aquat. Toxicol.* **15**, 83-98.

(14) Schüürmann, G., Somashekar, R.K., and Kristen, U. (1996) Structure-activity relationships for chloro- and nitrophenol toxicity in the pollen tube growth test. *Environ. Toxicol. Chem.* **15**, 1702-1708.

(15) Schüürmann, G., Flemmig, B., Dearden, J.C., and Cronin, M.T.D. (1997) CoMFA study of acute toxicity of nitrobenzenes to *Tetrahymena pyriformis*. In *Quantitative Structure-Activity Relationships in Environmental Sciences - VII* (Chen, F., and Schüürmann, G., Eds) pp 315-127, SETAC Press, Pensacola (FL), USA.

(16) Yuan, X., Lu, G., and Lang, P. (1997) QSAR Study of the toxicity of nitrobenzenes to river bacteria and Photobacterium phosphoreum. *Bull. Environ. Contam. Toxicol.* **58**, 123-127.

(17) Cronin, M.T.D., Gregory, B.W., and Schultz, T.W. (1998) Quantitative structure-activity analyses of nitrobenzene toxicity to Tetrahymena pyriformis. *Chem. Res. Toxicol.* **11**, 902-908.

(18) Kühn R., and Pattard, M. (1990) Results of the harmful effects of water pollutants to green algae (Scenedesmus subspicatus) in the cell multiplication inhibition test. *Wat. Res.* **24**, 31-38.

(19) Calamari D., Galassi, S., Setti, F. and Vighi, M. (1983) Toxicity of selected chlorobenzenes to aquatic organisms. *Chemosphere* **12**, 253-262.

(20) Lipnick, R.L. (1995) Structure-activity relationships. In *Fundamentals of aquatic toxicology*, 2nd edition (Rand, G.R., Ed.) pp 609-655, Taylor & Francis, London, UK.

(21) Schüürmann, G. (1998) Ecotoxic modes of action of chemical substances. In *Ecotoxicology* (Schüürmann, G., and Markert, B., Eds.) pp 665-749, John Wiley and Spektrum Akademischer Verlag, New York, USA.

(22) Washburn, P.C., and Di Giulio, R.T. (1989) Stimulation of superoxide production by nitrofurantoin, p-nitrobenzoic acid amd m-dinitrobenzene in hepatic microsomes of three species of freshwater fish. *Environ. Toxicol. Chem.* **8**, 171-180.

(23) Di Giulio, R.T., Washburn, P.C., Wenning, R.J., Winston, G.W., and Jewell, C.S. (1989) Biochemical responses in aquatic animals: A review of determinants of oxidative stress. *Environ. Toxicol. Chem.* **8**, 1103-1123.

(24) Lemaire, P., and Livingstone, D.R. (1994) Inhibition studies on the involvement of flavoprotein reductases in menadione- and nitrofurantoin-stimulated oxyradical production by hepatic microsomes of flounder

(Platichthys flesus). *J. Biochem. Toxicol.* **9**, 87-95.

(25) Lemaire, P., Matthews, A., Förlin, L., and Livingstone, D.R. (1994) Stimulation of oxyradical production of hepatic microsomes of flounder (Platichthys flesus) and perch (Perca fluviatilis) by model and pollutant xenobiotics. *Arch. Environ. Contam. Toxicol.* **26**, 191-201.

(26) Lackner, R. (1998) "Oxidative stress" in fish by environmental pollutants. In *Fish Ecotoxicology* (Braunbeck, T., Hinton, D.E., and Streit, B., Eds.) pp 203-224, Birkhäuser Verlag, Basel, Switzerland.

(27) Schultz, T.W., Sinks, G.D., and Cronin, M.T.D. (1997) Identification of mechanisms of toxic action of phenols to Tetrahymena pyriformis from molecular descriptors. In *Quantitative Structure-Activity Relationships in Environmental Sciences - VII* (Chen, F., and Schüürmann, G., Eds.) pp 329-342, SETAC Press, Pensacola (FL), USA.

(28) Altenburger, R., Bödecker, W., Faust, M., and Grimme, L.H. (1990) Evaluation of the isobologram method for the assessment of mixtures of chemicals. Combination of effect studies with pesticides in algal biotests. *Ecotoxicol. Environ. Saf.* **20**, 98-114.

(29) Hansch, C., Leo, A., and Hoekman, D. (1995) *Exploring QSAR. 2. Hydrophobic, electronic, and steric constants.* American Chemical Society, Washington (DC), USA.

(30) *CLOGP, version 4.61* (1999) Daylight Chemical Information Systems, Irvine (CA), USA.

(31) *SYBYL Molecular Modelling Software 6.4* (1998) Tripos Asociates Inc., St. Louis, Missouri, USA.

(32) Dewar, M.J.S., Zoebisch, E.G., Healy, E.F., and Stewart, J.J.P. (1985) AM1: A new general purpose quantum mechanical molecular model. *J. Am. Chem. Soc.* **107**, 3902-3909.

(33) *MOPAC 93, Revision 2* (1994) Fujitsu Limited, 9-3, Nakase 1-Chome, Mihama-ku, Chiba-city, Chiba 261, Japan, and Stewart Computational Chemistry, 15210 Paddington Circle, Colorado Springs, Colorado 80921, USA.

(34) Perrin, D.D., Dempsey, B., and Serjeant, E.P. (1981) *pK_a Prediction for Organic Acids and Bases*, Chapman and Hall, Cambridge, UK, 146 pp.

(35) Jafvert, C.T., Westall, J.C., Grieder, E., and Schwarzenbach, R.P. (1990) Distribution of hydrophobic ionogenic organic compounds between octanol and water: Organic acids. *Environ. Sci. Technol.* **24**, 1795-1803.

(36) Aries, R.E., Lidiard, D.P., and Spragg, R.A. (1991) Principal component analysis. *Chem. Br.*, 821-824.

(37) Marquardt, H., and Schäfers, S., Eds. (1994) *Lehrbuch der Toxikologie.* BI Wissen-schaftsverlag, Mannheim, Germany, p. 99.

(38) Penning, T.M., Burczynski, M.E., Hung, C.-F., C.Coull, K.D., Palackal, N.T., and Tsuruda, L.S. (1999) Dihydrodiol dehydrogenases and polycyclic aromatic hydrocarbon activation: Generation of reactive and redox active o-quinones. *Chem. Res. Toxicol.* **12**, 1-18.

(39) Roberts, D.W. (1995) Linear free energy relationships for reactions of electrophilic halo- and pseudohalobenzenes, and their application in prediction of skin sensitization potential for S_NAr electrophiles. *Chem. Res. Toxicol.* **8**, 545-551.

(40) Mekenyan, O., Roberts, D.W., and Karcher, W. (1997) Molecular orbital parameters as predictors of skin sensitization potential of halo- and pseudohalobenzenes acting as S_NAr electrophiles. *Chem. Res. Toxicol.* **10**, 994-1000.

(41) Aquatox Database, Release 1.12 (1990) BKH Consulting Engineers, Den Haag, The Netherlands. Cited after: Van Leeuwen, C.J., van der Zandt, P.T.J., Aldenberg, T., Verhaar, H.J.M., and Hermens, J.L.M. (1992) Application of QSARs, extrapolation and equilibrium partitioning in aquatic effects assessment. I. Narcotic industrial pollutants. *Environ. Toxicol. Chem.* **11**, 267-282.

(42) Veith G.D., and Broderius, S.J. (1987) Structure-toxicity relationships for industrial chemicals causing type (II) narcosis syndrome. In *QSAR in Environmental Toxicology - II*

(Kaiser, K.L.E., Ed.) pp 385-391, D. Reidel, Dordrecht, NL.

(43) AQUIRE (1992) Aquatic toxicity information retrieval, US EPA, ERL-Duluth, Duluth (MN), USA.

(44) McKim, J.M., Schmieder, P.C., Carlson, R.W., Hunt, E.P., and Niemi, G.J. (1987) Use of respiratory-cardiovascular responses of rainbow trout (*Salmo gairdneri*) in identifying fish acute toxicity syndromes. Part I. Pentachlorophenol, 2,4-dinitrophenol, tricaine methanesulfonate, and 1-octanol. *Environ. Toxicol. Chem.* **6**, 295-312.

(45) Schüürmann, G., Segner, H., and Jung, K. (1997) Multivariate mode-of-action analysis of acute toxicity of phenols. *Aquat. Toxicol.* **38**, 277-296.

Kapitel VII

Alterations of physiological energetics, growth and reproduction of Daphnia magna under toxicant stress.

Monika Knops, Rolf Altenburger und Helmut Segner
Aquatic Toxicology, 53, 79-90. (2001)

ELSEVIER

Aquatic Toxicology 53 (2001) 79–90

AQUATIC TOXICOLOGY

www.elsevier.com/locate/aquatox

Alterations of physiological energetics, growth and reproduction of *Daphnia magna* under toxicant stress

M. Knops, R. Altenburger, H. Segner *

Department of Chemical Ecotoxicology, UFZ Centre for Environmental Research, Permoserstrasse 15, D-04318 Leipzig, Germany

Received 11 May 2000; received in revised form 28 August 2000; accepted 31 August 2000

Abstract

The study investigates the relationship between changes in physiological energetics of organisms and alterations of growth, development and reproduction of *Daphnia magna*. Groups of primiparous daphnids were subjected to 8-day exposures to the heavy metals cadmium and copper or to the cationic surfactant, cetyltrimethylammonium bromide (CTAB). Energetic alterations were estimated from the measurement of oxygen consumption and feeding activity which was performed during the last 3 days of the exposure period and from the calculation of simplified carbon balances. The physiological effects were compared to effects on organismal growth and reproduction as obtained from 17-day exposure experiments. Toxicant exposure reduced weight and body length of daphnids indicating an impaired growth rate, but effects on total metabolic costs measured as weight-specific oxygen consumption could not be detected. Net carbon gain of individuals decreased in a concentration-dependent way for the tested chemicals reflecting effects on biomass of daphnids. In the case of cadmium and copper, reproduction ($\Sigma\, mx$: number of offspring per female of age x born during the time interval $x-1$ to x, summarised over the entire exposure period) and the estimate for the intrinsic rate of natural increase, derived from the 17-day exposure-experiment, were affected at concentrations comparable to the effect levels as observed for growth. In the case of copper, the concentrations affecting growth and reproduction were close to the 17-day LC_{50} value. CTAB caused a reduction in body length of primiparous daphnids whereas a decrease in the reproductive performance was not apparent. In conclusion, the chemicals did not change metabolic costs of exposed daphnids as it would be expected as a consequence of resistance or repair mechanisms, however, they induced alterations of SFG, growth, reproduction and intrinsic rate of natural increase. These alterations were chemical-specific. The fact that toxicant-related effects on growth and reproduction could not be linked to an elevated metabolic rate of daphnids may indicate that demand side effects occurred early during exposure — before the start of respirometric measurements — or that effects on growth were caused by an altered energy uptake. The results illustrate the importance of trade-off processes in regulating the distribution of energy among growth and reproduction of daphnids.

Keywords: Daphnia; Oxygen consumption; Sublethal effects; Growth; Reproduction

* Corresponding author. Present address: Centre for Fish and Wildlife Health, University of Berne, Länggass-Strasse 122, CH-3012 Berne, Switzerland. Tel.: +41-31-6312441; fax: +41-31-6312611.

E-mail address: helmut.segner@itpa.unibe.ch (H. Segner).

0166-445X/01/$ - see front matter

PII: S0166-445X(00)00170-3

1. Introduction

Disturbance of the homeostasis of an organism leads to compensatory, adaptive and finally pathological processes which are mostly energy-demanding. Therefore, the metabolic rate of an organism should be increased under toxic stress (Calow, 1989). Due to the limited energy resources of organisms, the additional metabolic costs result in a reallocation of energy resources and may only be met at the expense of other energy-demanding processes (Beyers et al., 1999) or by an enhanced energy intake.

Information about the metabolic rate of individuals can be obtained from the analysis of oxygen consumption. With this methodological approach, effects of toxicants on metabolic costs of the whole organism can be measured. Usually, three categories of metabolism are distinguished, standard metabolic rate, routine metabolic rate, and active metabolic rate, depending on the extend to which spontaneous muscular activity is eliminated, or kept constant at a high level (Heath, 1995). The routine metabolic rate of fed organisms integrates various physiological processes which may not react in the same way to chemical stress. Therefore, toxicant-induced alterations of oxygen consumption of individuals depend on the contribution of the involved processes to the total metabolic rate.

Results of oxygen consumption records can be integrated with measurements of absorbed food in energy balance equations and provide information about energy resources for growth and reproduction. Calculating energy available for somatic and reproductive growth as difference between energy income and expenditure ('Scope for Growth') is discussed as a tool to link effects on (sub-)organismal level to the fitness variables survivorship, developmental rate and fecundity which affect population dynamics (Calow and Sibly, 1990).

Understanding the relation between toxicant effects at different levels of biological organisation is of crucial importance in ecotoxicological research (Maltby and Calow, 1989). However, the prediction of effects at higher levels of biological hierarchy from effects observed at lower levels is complicated by the fact that trade-offs occur between life-history traits. Trade-off means that a secondary response can be considered as (partly) compensatory to the direct toxic effect (van Straalen and Kammenga, 1998): for instance, direct effects on organismal growth are not necessarily reflected in altered population dynamics, if the effect on somatic growth is indirectly compensated by an enhanced energy transfer to reproduction. Another problem in extrapolation arises from the fact that responses at different levels of biological organisation show intrinsically different characteristics. Going from the cell to the population or community level implies that emerging properties are studied with each an increasing degree of complexity. As a consequence of these considerations, simultaneous measurements of physiological processes and fitness parameters provide an important tool to understand mechanisms of toxicological effects.

In our study we recorded oxygen consumption and feeding activity of primiparous daphnids which had been exposed to toxicants during their juvenile period. These data were used to calculate simplified carbon balances as an estimate of the metabolic costs of the toxic response. The results of the respirometric experiments were compared with effects on growth and reproductive performance, as obtained from 17-day exposure experiments.

Main objectives of the present study were

1. to assess effects of chemical exposure on metabolic costs of *Daphnia magna*;
2. to consider the relationship between physiological responses of organisms induced by chemical stress and alterations of reproductive effort and the intrinsic rate of natural increase as a measure of fitness.

2. Material and methods

2.1. Culture of D. magna

Animals were reared individually in artificial medium (ADaM) prepared according to Klüttgen et al. (1994). They were fed three times a week with *Scenedesmus vacuolatus* harvested from syn-

chronous cultures as described in Altenburger et al. (1990), centrifuged and resuspended in ADaM. Food was supplemented by a suspension of yeast extract once a week. *D. magna* cultures were kept at 20°C with a photo-period of 16:8 h and a light intensity of ~ 3 W/m^2.

2.2. Chemicals

Cetyltrimethylammonium bromide (CTAB; > 99%) and copper sulfate ($CuSO_4 \cdot 5H_2O$; > 99%) were purchased from Merck (Germany), cadmium chloride ($CdCl_2 \cdot H_2O$; > 98%) from Riedel-de-Haen (Germany). At the beginning of each experiment, concentrated geometrical dilution series of the chemicals were prepared in bi-distilled water and stored at 4°C. Aliquots of these stock solutions were diluted with aerated *Daphnia* medium to obtain the final test concentrations.

2.3. Effects on carapace length and reproduction (17-day exposure-experiments)

For the 17-day exposure-experiments as well as for the other physiological experiments, described below, light intensity was reduced to ~ 0.7 W/m^2 to restrict the impact of algal photosynthesis. Daphnids were fed daily with a total algal volume of $9 \cdot 10^9$ fl (fl = 10^{-15} l) per vessel corresponding to a final concentration of $\sim 5.6 \cdot 10^5$ autospores per ml (~ 0.07 mg C/daphnid/day). Autospores were obtained from synchronous algal cultures after mitotic cell division. Algal biovolume was determined using a cell analyser system (Casy II, Reutlingen, Germany).

Each chemical was tested using seven concentrations (10 daphnids/540 ml) without replicates. (Nominal) concentrations tested were in the range: 17–52 μg/l CTAB, 4.1–12 μg/l Cu^{2+}, and 0.74–8.4 μg/l Cd^{2+}, respectively. In previous lethality tests, using the same exposure conditions except for food ration which was supplied only once at the beginning of an experiment, EC_{50} (48 h) values for neonates (< 48 h) were determined. The values were 50 μg/l (CTAB), 0.47 mg/l (Cd^{2+}), and 11 μg/l (Cu^{2+}). The medium was renewed three times a week. Dead animals and released neonates were counted and removed daily (except day 16 of exposure). Age-specific survival (lx) and reproduction (mx) were recorded over a period of 17 days. The exposure period of 17 days should regularly include the release of the first and second brood. Therefore, possible adverse effects on developmental time and consequences for the intrinsic rate of natural increase were integrated by the selected approach. As shown by van Leeuwen et al. (1985), time to maturity contributes to the intrinsic rate of natural increase to a great extend. Reproduction was determined as number of offspring per female of age x, born during the time interval $x-1$, summarised over the entire exposure period ($\Sigma\, mx$). The intrinsic rate of natural increase (r) was calculated using a numerical technique as described by Carey (1993). Carapace length of daphnids was measured from the top of the head to the base of the spine on days 8 and 17 of exposure using an ocular micrometer. Concentration-response functions were estimated using the logistic sigmoidal model equation (Figure Print software, Biosoft, UK) to calculate EC_{10} values for the different endpoints.

2.4. Effects on oxygen consumption, feeding activity and net carbon gain (8-day exposure-experiments)

2.4.1. Exposure conditions

A total of 150 neonates (< 24 h) were divided between 15 glass flasks containing 540 ml Daphnia medium (six controls) and three toxicant concentrations with three replicates, respectively. Nominal concentrations used for each chemical were:

CTAB: 36–43–52 μg/l
Cadmium (Cd^{2+}): 3.7–5.6–8.4 μg/l
Copper (Cu^{2+}): 6.0–7.8–10 μg/l

Exposure conditions were basically the same as described for the 17-day exposure-experiments above. Daphnids were fed daily with a total algal volume of $9 \cdot 10^9$ fl per vessel. Dead animals were removed daily. Toxicant concentrations were renewed on days 3 and 5 of exposure. Data for physiological energetics (oxygen consumption, feeding activity) were recorded for ten individuals over a period of 3 days, beginning on day 5 of exposure, when daphnids usually started to de-

posit eggs into their brood pouch. Measurements were performed with three controls and duplicates of each test concentration. Rearing conditions were the same as used for pre-exposure despite that the daily food ration was reduced to $3.5 \cdot 10^9$ fl per vessel, which meets the demand of 10 daphnids. The reduction in algal biomass was intended to limit photosynthetic oxygen production. On day 8, daphnids from all treatments were collected, dried at 60°C for 6 h and weighed (Sartorius RC 210 P, Göttingen, Germany).

2.4.2. Oxygen consumption

Oxygen consumption was continuously recorded using the closed circuit respirometer system Micro-Oxymax™ (Columbus Instruments, OH), supplied with a fuel cell. The O_2 gas concentration in the headspace of the test chambers was measured periodically by pumping air from the headspace through the sensor. Changes in gas concentration were used to calculate oxygen consumption. As test chambers, the same glass flasks as for pre-exposure were used, despite that they were provided with a small silicon septum at their neck. Through this arrangement, food could be injected without disturbing measurements. A measuring period of ~ 3 days (65 h) was selected to integrate alterations of metabolic activity during the day. To calculate effects of chemical exposure on total metabolic costs, cumulative oxygen consumption, determined as µl O_2 per ten individuals per 65 h (V_{O_2}), was corrected for algal photosynthesis. Data were then expressed as weight-specific oxygen consumption (µl O_2/mg dry weight/h) with reference to dry weight values determined at the end of the exposure period. As daphnids were fed with living algal cells during the measurements the impact of the algae on the recorded changes of oxygen concentrations in the headspaces had to be taken into consideration. For a correction for oxygen production due to photosynthesis a mean physiologically active algal biovolume was estimated assuming that feeding activity of daphnids results in a linear decrease in algal volume. A regression equation between algal volume and oxygen production was used which was established at similar conditions as used for the exposure experiments. For controls, oxygen consumption of daphnids was underestimated by about 32% due to algal oxygen production. This observation was verified by a control experiment using a low concentration of the photosynthesis inhibitor atrazine (0.5 µM) which is well below the chronic NOEC stated for daphnids (Rudolph and Boje, 1986). This experiment suggested that oxygen consumption of daphnids was underestimated by $\sim 31\%$, because oxygen consumption calculated for Daphnia–algae-systems without photosynthesis inhibition were only 69% of that obtained in the presence of atrazine. For Daphnia–algae–systems, exposed to copper, cadmium or CTAB, respectively, higher mean physiologically active algal biovolumes and hence higher values for photosynthetic oxygen production were calculated corresponding to the degree of feeding inhibition of daphnids. Furthermore, in the case of CTAB, these values were corrected for observed toxicant effects on algal photosynthesis by multiplying with 0.93 (36 µg/l CTAB), 0.79 (43 µg/l CTAB), and 0.68 (52 µg/l CTAB).

Cumulative oxygen consumption, determined as µl O_2 per ten individuals per 65 h (V_{O_2}), was converted into carbon losses using the following equation assuming that the respiratory quotient (RQ) is 1 and that the metabolism is strictly aerobic:

$$\text{Carbon losses (mg)} = V_{O_2} \cdot 0.001 \cdot (V_m)^{-1} \cdot MG_C$$

$V_m = 22.4$ ml/mmol (mol volume); $MG_C = 12$ mg/mmol (molecular weight of carbon)

2.4.3. Feeding activity

Feeding activity was determined simultaneously with oxygen consumption as the difference between the sum of the supplied algal volume and the algal biovolume remaining in the test chambers at the end of an experiment. Particles, ranging from 1.5 to 15 µm in diameter as determined by the particle analyser, were considered as source of food. The calculated values were converted into carbon uptake using a correlation factor between algal volume and TOC-content. This factor was determined by constructing a regression of TOC-content versus algal volume from a geometrical dilution series of an algal suspension in distilled water (Elementar High TOC). For the calculation,

it was assumed that toxicants had no impact on the carbon content of the algae.

2.4.4. Calculation of carbon balances

In this study, scope for growth was assessed as net carbon gain. This parameter was calculated as the difference between carbon uptake and carbon losses. Results were presented as percent deviation from the mean value of the control vessels. As described above, preliminary experiments were performed, in which daphnids together with algae were exposed to the photosynthesis inhibitor atrazine to investigate the impact of algal photosynthesis. The decrease in algal biovolume, calculated for Daphnia–algae systems. Without atrazine was found to be only 80% of the value estimated for photosynthesis-inhibited systems. Therefore, feeding activity of daphnids was assumed to be underestimated by $\sim 20\%$. As not only oxygen consumption but also feeding activity was underestimated, the difference between carbon uptake and carbon losses was less affected and we therefore neglected the impact of algal photosynthesis in order to simplify the SFG-calculations.

3. Results

3.1. Effects of toxicants on carapace length and reproduction (17-day exposure-experiments)

The effects of exposure to CTAB, copper and cadmium on carapace length of *D. magna* are shown in Fig. 1A–C. All toxicants caused a reduction in body length of daphnids after 8-day exposure to the higher concentration levels. Concentration–response functions were described using the logistic sigmoidal model equation to estimate the EC_{10} for the parameters, which were determined in the 17-day experiments (Table 1). Regarding carapace length, EC_{10} values for CTAB were 42 and 65 μg/l after 8 and 17 days of exposure. The 10% effect levels for copper were 11 μg/l at both observation times. For cadmium, EC_{10} concentration of ~ 6.3 μg/l on day 8 and 7.3 μg/l on day 17 were calculated.

Effects of CTAB on reproduction and intrinsic rate of natural increase are presented in Fig. 1D. Exposure to the highest CTAB concentration (52 μg/l) caused lethal effects (50% at the end of the observation period of 17 days). Reproduction ($\Sigma\, mx$) of toxicant-exposed daphnids was elevated especially at higher CTAB concentrations compared to the corresponding control value. As a result, the values estimated for the intrinsic rate increased, except for the highest test concentration where survivorship of daphnids was adversely affected. The results of the reproduction test performed with copper are shown in Fig. 1E. Survival was adversely affected at concentrations ≥ 5.9 μg/l (17-day LC_{50}: 9.4 μg/l copper, Table 1). The estimated values for the intrinsic rate of natural increase decreased concentration-dependent (EC_{10} 7.5 μg/l, Table 1). A distinct reduction in reproduction ($\Sigma\, mx$) was found only at 10 μg/l copper. Due to lethal effects, the decrease in the intrinsic rate of natural increase was pronounced at higher test concentrations. At copper concentrations below the LC_{50} (17 days) reproduction was not adversely affected. Exposure to cadmium caused a distinct reduction in reproduction at concentrations ≥ 3.7 μg/l (Fig. 1F). At this concentration level the intrinsic rate of natural increase was affected as well. EC_{10} values of 3.1 and 5.6 μg/L cadmium were calculated for reproduction ($\Sigma\, mx$) and the intrinsic rate of natural increase, respectively (Table 1). Lethality was markedly elevated at the highest test concentration (8.4 μg/l) and a LC_{50} value of 7.3 μg/l was estimated for the exposure period of 17 days.

3.2. Effects on oxygen consumption, feeding activity and net carbon gain (8-day exposure-experiments)

The mean weight-specific oxygen consumption of daphnids, which was derived from respirometer recordings performed over a period of 65 h from days 5 to 8 of exposure, did not change as a result of chemical stress (Table 2).

The individual oxygen consumption, which corresponds to the oxygen consumption of ten daphnids without standardisation to their weight, and hence the calculated carbon losses, decreased after

8-day exposure to CTAB, copper and cadmium (Fig. 2). Furthermore, toxicant exposure caused a concentration-dependent decrease in carbon uptake, as estimated from algal biovolume ingestion. This is illustrated in Fig. 2.

The net carbon gain (SFG), which is equivalent to the difference between carbon uptake (open bars in Fig. 2) and carbon losses (hatched bars, Fig. 2) decreased, when daphnids were exposed to the chemicals for 8 days. This was mainly due to a reduction in absolute carbon uptake. A reduction in net carbon gain of about more than 10% was determined for organisms kept at concentrations ≥ 43 μg/l CTAB and for those which were exposed to the highest copper and cadmium levels as shown in Fig. 3.

In order to link changes in the organismal energetics to growth and reproduction, effects on SFG were compared to alterations in dry weight of the same test organisms and to effects on egg production as a measure for energy transfer to reproduction (Σmx including aborted eggs) obtained from the 17-day exposure-experiments. Toxicant effects on SFG of daphnids covaried with changes in dry weight of stressed individuals (Fig. 3), although deviations from control values were usually higher for the latter parameter. When daphnids were exposed to the heavy metals

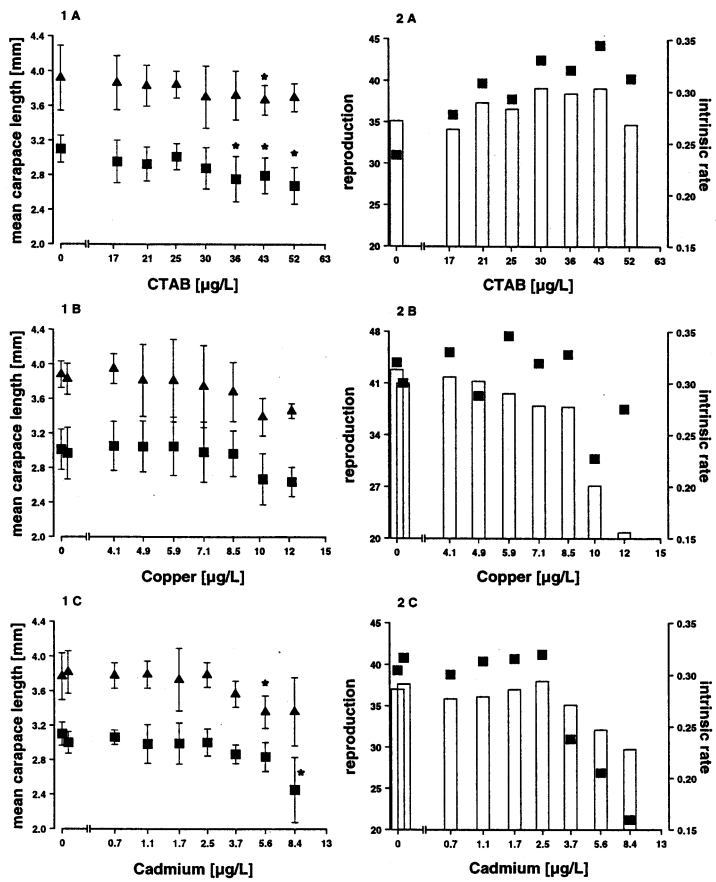


Fig. 1. (A–C) Carapace length (mm), expressed as means ± S.D., after 8 days (square) and 17 days (triangle) exposure to CTAB (A), copper (B) and cadmium (C). Values, which differ significantly from control ($P < 0.05$), are marked with an asterix. (D–F) Reproduction (Σmx: number of offspring per female of age x born during the time interval $x-1$ to x, summarised over the entire exposure period; solid squares) and mean intrinsic rate of natural increase values (open bars) for *Daphnia magna* exposed to different CTAB (D), copper (E) and cadmium concentrations (F) for 17 days.

Table 1
Results of the regression model equations (logistic sigmoidal function) estimating the concentration–response functions for the different effect parameters obtained in the 17-day exposure-experiments and the derived EC_{10} values[a]

Toxicant	Parameter	$Y = \text{Min} + (\text{Max} - \text{Min})/(1 + \exp(-K^*(X - X50)))$			EC_{10} (μg/l)
		Max	K	$X50$ (S.E.)	
CTAB	Length (8 days)	100.0 (fix)	−0.036	103.0 (14.8)	42.0
	Length (12 days)	100.0 (fix)	−0.069	90.3 (13.0)	58.5
	Length (14 days)	100.0 (fix)	−0.045	105.1 (17.0)	56.3
	Length (17 days)	100.0 (fix)	−0.030	138.5 (37.7)	65.3
	Σ *mx* (17 days)	–	–	–	–
	r (17 days)	–	–	–	–
	Lethality (17 days)	100.0 (fix)	0.247	52.0 (1.0)	
Copper	Length (8 days)	108.1	−0.204	19.0 (4.0)	11.1
	Length (12 days)	108.1 (fix)	−0.147	21.8 (2.8)	10.9
	Length (15 days)	108.1 (fix)	−0.147	22.4 (1.3)	11.5
	Length (17 days)	108.1 (fix)	−0.145	22.0 (2.7)	10.9
	Σ *mx* (17 days)	114.1	−0.199	16.8 (7.8)	10.2
	r (17 days)	100.0 (fix)	−0.503	11.9 (0.3)	7.5
	Lethality (17 days)	100.0 (fix)	0.508	9.4 (0.3)	
Cadmium	Length (8 days)	100.0 (fix)	−0.380	12.1 (0.6)	6.3
	Length (12 days)	100.0 (fix)	−0.240	14.6 (1.9)	5.4
	Length (15 days)	100.0 (fix)	−0.327	12.7 (0.7)	6.0
	Length (17 days)	100.0 (fix)	−0.267	15.5 (3.1)	7.3
	Σ *mx* (17 days)	100.0 (fix)	−0.425	8.2 (0.8)	3.1
	r (17 days)	100.0 (fix)	−0.333	12.2 (1.3)	5.6
	Lethality (17 days)	100.0 (fix)	0.753	7.3 (0.5)	

[a] Abbreviations: Max, maximal effect; Min, minimal effect, always kept at '0'; K, $X50$, estimated parameters of the logistic sigmoidal function, S.E., standard error; (fix), denotes that the maximum effect has been set.

cadmium and copper, the reduced SFG and the reduced dry weight of daphnids, observed after 8-day exposure, covaried with a decrease in reproduction in the 17-day exposure-experiments (Fig. 3). For CTAB however, no relationship could be found between effects on the carbon amount available for biomass production and the cumulative egg number (Fig. 3).

4. Discussion

With respect to the objectives of the study the following answers can be deduced from the experimental results:

- The weight-specific oxygen consumption as a measure for the metabolic rate was not altered after extended exposure (5–8 days) to toxicant stress, although individual net carbon gain decreased in a concentration-dependent way. This finding does not support the view that whole-organismal metabolic costs are elevated due to chemical stress.
- In case of CTAB, reduction in body length and net carbon gain of primiparous daphnids were not associated with negative effects on reproduction and the intrinsic rate of natural increase in contrast to the results obtained for cadmium and copper. Resource allocation between growth, reproduction and survival seems to be affected in a chemical-specific way following chronic exposure.

The observation that the weight-specific oxygen consumption of daphnids is not affected by toxicants after longer exposure periods is in the case of cadmium in agreement with findings of other studies (Barber et al., 1990, 1994). Likewise, a reduction in body weight (Bodar et al., 1988a,

Table 2
Effect of 8-day exposure to CTAB, copper and cadmium on weight-specific oxygen consumption of daphnids (μl O_2/mg dry weight/h) calculated for ten individuals during the last 65 h of the exposure period[a]

Toxicant	Conc. (μg/l)	Oxygen consumption (μl O_2/mg/h)% of control	
CTAB	Control	5.09 (4.63; 5.35; 5.29)	
	36	4.97 (4.80; 5.14)	−2
	43	5.02 (4.67; 5.37)	−1
	52	4.94 (4.67; 5.21)	−3
Copper	Control	4.94 (4.80; 4.84; 5.20)	
	6.0	5.27 (–)	7
	7.8	4.88 (4.51; 5.25)	−1
	10	5.31 (–)	7
Cadmium	Control	5.37 (5.13; 5.39; 5.58)	
	3.7	5.22 (5.27; 5.17)	−3
	5.6	5.09 (5.23; 4.94)	−5
	8.4	5.25 (5.17; 5.33)	−2

[a] Results are presented as absolute values (means, single values in brackets) and as percent deviation from controls (mean of three control replicates)

Baird et al., 1990) and length (Baird et al., 1990; Klüttgen and Ratte, 1994) has been reported for daphnids chronically exposed to cadmium as well as a decline in the assimilation rate (Bodar et al., 1988a). In principle, there are three possible explanations for an unchanged metabolic rate under stress: (1) additional costs associated with resistance/repair processes are masked by other toxicant effects, (2) these energy demands are too small compared to whole-metabolic costs to be evident, (3) there are, at least during the investigated exposure period, no additional costs due to chemical stress.

Masking of stress-specific energetic costs may result from reduced metabolic costs associated with food acquisition, digestion and growth-components leading to the heat increment of feeding (SDA) (Widdows and Hawkins, 1989) — or reduced energetic costs for locomotory activity. Therefore, an additional energy demand due to resistance/repair mechanisms in certain tissues may not alter oxygen consumption of individuals when feeding activity is affected by the toxicant. According to Barber et al. (1990) a constant respiration rate despite a reduction in assimilation possibly indicates increased energy demands for maintenance. Elevated costs for maintenance may also be masked by reduced locomotory activity. Heath (1995) suggested that exposure of fish to sublethal copper concentrations causes increased maintenance costs resulting in higher metabolic rates in certain non-muscular tissues while sponta-

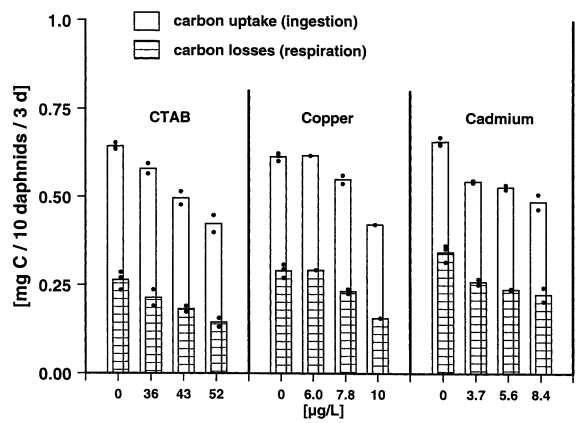


Fig. 2. Effect of 8-day exposure to CTAB, copper and cadmium on carbon uptake (open bars) and carbon losses (hatched bars) of *D. magna* calculated for ten individuals during the last 65 h of the exposure period. Carbon uptake was derived from the decrease in algal volume using a correlation factor between algal volume and TOC-content determined for unexposed algae. Carbon losses were deduced from cumulative oxygen consumption assuming that metabolism was aerobic and RQ was 1. Bars represent mean values of triplicates (controls) or duplicates (toxicant concentrations, except for 6.0 and 10 μg/l copper, where only one replicate could be evaluated). The circles represent the corresponding single values.

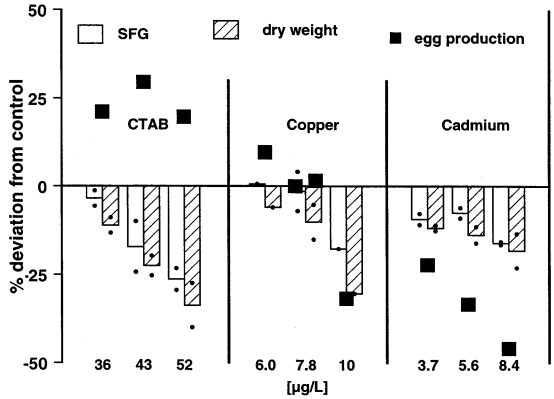


Fig. 3. Sublethal effects of CTAB, copper and cadmium on dry weight (hatched bars) and net carbon gain (scope for growth, open bars) of *D. magna* (8-day exposure-experiments). Results are demonstrated as percent deviation from the mean values of three controls. The data points (bars) represent mean effects obtained for duplicates consisting of ten individuals each, except for 6.0 and 10 µg/l copper, where single values are presented. The circles represent the corresponding single values. In comparison, effects on egg production ($\Sigma\, mx$ including aborted eggs: number of eggs and offspring per female of age x released during the time interval $x-1$ to x, summarised over the entire exposure period; solid squares) derived from the 17-day exposure experiments are depicted.

neous muscular activity becomes depressed. Such a masking effect may not be relevant under laboratory conditions where food is abundant and no predators are present, but could be of disadvantage in the field.

Metabolic costs involved in the induction of specific protective processes might be negligible compared to whole body energy expenditure. For instance, costs for metallothionein synthesis which is a prototype of cellular response to metal exposure, are estimated to account for less than 5% of total metabolic costs in daphnids (Barber et al., 1990). Wilson (1996) stated that the increase in gill protein synthesis, which was observed in fish after prolonged exposure to acid and aluminium, represents only $\sim 2.3\%$ of whole body protein synthesis and, therefore, the additional energy demand for metal-induced metallothionein synthesis will probably be small, provided that metallothionein synthesis is the major energy-demanding process under sublethal metal exposure. Lyndon and Houlihan (1998) supported the hypothesis that energetic costs associated with the adaption of branchial protein metabolism to environmental stress are relatively small compared to whole metabolic costs of fish. Likewise, Alsop et al. (1999) found no effect of zinc exposure on either resting and routine metabolic rate or growth of rainbow trout, which had acclimated to the metal as assessed by zinc tolerance (96-h LC_{50}). These authors concluded that there is no evidence for long-term costs resulting from the metal acclimation processes. However, they stressed that initial costs would not have been recorded as acclimation usually occurs within 5–15 days of sublethal exposure and measurements were performed after 30 days. With respect to *Daphnia*, acclimation was reported to develop within 7–20 h (LeBlanc, 1982; Stuhlbacher et al., 1992), therefore initial energy demands may also have been missed in our study where respirometric measurements were performed between days 5 and 8 of exposure.

In order to estimate SFG, we have calculated carbon uptake and carbon losses for groups of 10 individuals. The results suggested that the reduction in SFG was mainly due to supply-side effects, i.e. a decrease in ingestion. Because the maximal ingestion rate depends on body length (see McCauley et al., 1990) this effect might be to some extend the result of a delay in growth, therefore this interpretation has to be judged with caution. However, a reduction in SFG due to a decrease in energy absorbed has also been reported for *Gammarus* exposed to zinc (Naylor et al., 1989; Maltby and Naylor, 1990) or to low pH (Naylor et al., 1989).

The reduction in clutch size and clutch mass of daphnids observed under cadmium stress is supposed to be mainly due to effects on body size (Baird et al., 1990). As shown for CTAB, effects on growth did not necessarily correlate with a decrease in reproduction. DeCoen (1998) determined effect thresholds concerning body length and reproduction for several toxicants and distinguished three groups: (1) toxicants with identical threshold values for adult growth and reproduction (2) toxicants with a higher threshold for reproduction than for body size (3) toxicants where an effect on reproduction was detected at

concentrations lower than the LOEC for length. According to DeCoen (1998), cadmium was a representative of the first group, whereas the surfactant LAS matches the criterion of the second class. In our study, reproduction appeared to be a sensitive parameter with respect to chronic cadmium stress, whereas the surfactant CTAB affected mainly juvenile growth, which is in agreement with the observations of DeCoen (1998).

As stated by Forbes and Forbes (1994) some organisms may maintain the reproductive output at the expense of growth, i.e. will uncouple SFG from reproduction. In contrast, Lampert and Trubetskova (1996) postulated that the juvenile growth rate could be used as a predictor for effects on the intrinsic rate of natural increase. This statement is based on the assumption that the fraction of total production allocated to reproduction is constant and does not depend on absolute growth.

In the described experiments we did not consider clutch mass or energy/carbon content of produced eggs. However, results of a subsequent experiment indicate that first-brood neonates of CTAB-exposed daphnids had a reduced ability to resist starvation compared to individuals born under unstressed conditions. This observation could possibly be explained by lower energy reserves of neonates. Therefore, reproduction might not only be maintained at the expense of growth but also at the expense of mass or lipid content of individual eggs under CTAB exposure.

A modification of life-history traits has been reported from other toxicological studies as well, e.g. when daphnids were exposed to fish-exuded substances (Stibor, 1992; Dawidowicz and Loose, 1992; Weider and Pijanowska, 1993). Daphnids raised in fish-treated water show a smaller body size at first reproduction. Stibor and Machácek (1998) suggested that the exudate-induced decline in body length and the increase in reproductive effort of daphnids are facultative changes as they are not associated with changes in size-specific assimilation and respiration rates. According to Dawidowicz and Loose (1992) the total biomass production decreased and therefore lower investments in somatic growth are not completely compensated by an elevated resource allocation to reproduction. Dry weights of eggs (Dawidowicz and Loose, 1992) and neonates (Sakwinska, 1998) are reduced when daphnids are raised in fish-treated water. Larger broods with smaller neonates compared to unstressed individuals are also reported for *D. magna* exposed to low cadmium concentrations (Bodar et al., 1988b). A trade-off between brood quantity and quality has often been described as a response to changing food conditions and/or higher animal density (Enserink et al., 1990; Cox et al., 1992; Gliwicz and Giusande, 1992; Enserink et al., 1995; Trubetskova and Lampert, 1995; Cleuvers et al., 1997; Goser, 1997). However, under these circumstances the quality of neonates increases under stress: except for extremely low food levels, daphnids raised under stress produce fewer but larger eggs and offspring containing more lipids. These offspring have a higher survival probability under starvation conditions (Tessier et al., 1983; Cowgill et al., 1984; Gliwicz and Giusande, 1992; Cleuvers et al., 1997). The underlying mechanism is not known, but body storage of individuals — defined as somatic mass adjusted to body length — is probably not the clue to control resource allocation (Glazier, 1998). Nevertheless, the consequences of a reduced energy uptake due to limited food availability may be different from effects resulting from a toxicant-induced decrease in food intake, although the energy budget is affected in a similar way.

A potential disadvantage of life-table experiments is that they do not integrate effects on quality of neonates (Daniels and Allan, 1981). Hence, comparing effects on the SFG of individuals with effects on the intrinsic rate of natural increase may not always be a suitable approach to understand the ecological meaning of physiological stress responses.

In conclusion, the results of the study show that exposure of daphnids to sublethal concentrations of CTAB, copper or cadmium during extended periods of their life span does not cause measurable additional metabolic costs at the organismal level. The metabolic rate of daphnids was not altered, although acclimation is known to occur with respect to the heavy metals tested. Further

investigations are required to decide whether energetic demands due to stress responses are small or whether possible additional costs are masked by other effects. Although measurable metabolic costs were not altered, growth and SFG of daphnids declined for all chemicals investigated. Reproduction and intrinsic rate of natural increase showed a chemical-specific response: exposure to cadmium and copper induced a decrease in these parameters whereas CTAB led to a (slight) increase. The diverse response pattern of the various physiological indices to toxic stress illustrates the importance of trade-off processes. Thus, future studies should investigate more in detail the factors regulating the allocation of energy between different physiological processes.

Acknowledgements

The authors thank Dr J. Flachowsky and P. Fiedler for TOC analysis.

References

Alsop, D.H., McGeer, J.C., McDonald, D.G., Wood, C.M., 1999. Costs of chronic waterborne zinc exposure and the consequences of zinc acclimation on the gill/zinc interactions of rainbow trout in hard and soft water. Environ. Toxicol. Chem. 18, 1014–1025.

Altenburger, R., Bödeker, W., Faust, M., Grimme, L.H., 1990. Evaluation of the isobologram method for the assessment of mixtures of chemicals. Combination effect studies with pesticides in algal biotests. Ecotox. Environ. Saf. 20, 98–114.

Baird, D.J., Barber, I., Calow, P., 1990. Clonal variation in general responses of *Daphnia magna* Straus to toxic stress. I. Chronic life-history effects. Funct. Ecol. 4, 399–407.

Barber, I., Baird, D.J., Calow, P., 1990. Clonal variation in general responses of *Daphnia magna* Straus to toxic stress. II. Physiological effects. Funct. Ecol. 4, 409–414.

Barber, I., Baird, D.J., Calow, P., 1994. Effect of cadmium and ration level on oxygen consumption, RNA concentration and RNA–DNA ratio in two clones of *Daphnia magna* Straus. Aquat. Toxicol. 30, 249–258.

Beyers, D.W., Rice, J.A., Clements, W.H., Henry, C.J., 1999. Estimating physiological cost of chemical exposure: integrating energetics and stress to quantify toxic effects in fish. Can. J. Fish. Aquat. Sci. 56, 814–822.

Bodar, C.W.M., van der Sluis, I., Voogt, P.A., Zandee, D.I., 1988a. Effects of cadmium on consumption, assimilation and biochemical parameters of *Daphnia magna*: possible implications for reproduction. Comp. Biochem. Physiol. C 90, 341–346.

Bodar, C.W.M., van Leeuwen, C.J., Voogt, P.A., Zandee, D.I., 1988b. Effect of cadmium on the reproduction strategy of *Daphnia magna*. Aquat. Toxicol. 12, 301–310.

Calow, P., 1989. Physiological ecotoxicology; Theory, practice and application. In: Løkke, H., Tyle, H., Bro-Rasmussen, F., (Eds.), 1st European conference on ecotoxicology. A SECOTOX regional conference on testing, prediction and validation of pathways, fate and effects of chemicals in the environment, 17–19 October 1988, Copenhagen, Denmark, pp. 23–35.

Calow, P., Sibly, R.M., 1990. A physiological basis of population processes: ecotoxicological implications. Funct. Ecol. 4, 283–288.

Carey, J.R., 1993. Applied Demography for Biologists with Special Emphasis on Insects. Oxford University Press, Oxford.

Cleuvers, M., Goser, B., Ratte, H.T., 1997. Life-strategy shift by intraspecific interaction in *Daphnia magna*: change in reproduction from quantity to quality. Oecologia 110, 337–345.

Cowgill, U.M., Williams, D.M., Esquivel, J.B., 1984. Effects of maternal nutrition on fat content and longevity of neonates of *Daphnia magna*. J. Crustacean Biol. 4, 173–190.

Cox, E.J., Naylor, C., Bradley, M.C., Calow, P., 1992. Effect of differing maternal ration on adult fecundity and offspring size in laboratory cultures of *Daphnia magna* Straus for ecotoxicological testing. Aquat. Toxicol. 24, 63–74.

Daniels, R.E., Allan, J.D., 1981. Life table evaluation of chronic exposure to a pesticide. Can. J. Fish. Aquat. Sci. 38, 485–494.

Dawidowicz, P., Loose, C.J., 1992. Metabolic costs during predator-induced diel vertical migration of *Daphnia*. Limnol. Oceanogr. 37, 1589–1595.

DeCoen, W.M.I., 1998. Study of the energy metabolism and DNA damage of the waterflea *Daphnia magna* Straus under toxic stress and the relation with population dynamics. PhD Thesis University of Ghent, Belgium.

Enserink, E.L., Luttmer, W., Maas-Diepeveen, H., 1990. Reproductive strategy of *Daphnia magna* affects the sensitivity of its progeny in acute toxicity tests. Aquat. Toxicol. 17, 15–25.

Enserink, E.L., Kerkhofs, M.J.J., Baltus, C.A.M., Koeman, J.H., 1995. Influence of food quantity and lead exposure on maturation in *Daphnia magna*; evidence for a trade-off mechanism. Funct. Ecol. 9, 175–185.

Forbes, V.E., Forbes, T.L., 1994. Ecotoxicology in Theory and Practice. Chapman and Hall, London.

Glazier, D.S., 1998. Does body storage act as a food-availability cue for adaptive adjustment of egg size and number in *Daphnia magna*? Freshwater Biol. 40, 87–92.

Gliwicz, Z.M., Giusande, C., 1992. Family planning in *Daphnia*: resistance to starvation in offspring born to mothers grown at different food levels. Oecologia 91, 463–467.

Goser, B., 1997. Dichteabhängige Änderungen der Entwicklung und Reproduktion bei Cladoceren. PhD Thesis Technical University of Aachen, Germany.

Heath, A.G., 1995. Water Pollution and Fish Physiology, 2nd edn. Lewis Publishers, Boca Raton, FL.

Klüttgen, B., Ratte, H.T., 1994. Effects of different food doses on cadmium toxicity to *Daphnia magna*. Environ. Toxicol. Chem. 13, 1619–1627.

Klüttgen, B., Dülmer, U., Engels, M., Ratte, H.T., 1994. ADaM, an artificial freshwater for the culture of zooplankton. Water Res. 28, 743–746.

Lampert, W., Trubetskova, I., 1996. Juvenile growth rate as a measure of fitness in *Daphnia*. Funct. Ecol. 10, 631–635.

LeBlanc, G.A., 1982. Laboratory investigation into the development of resistance of *Daphnia magna* (Straus) to environmental pollutants. Environ. Pollut. Ser. A 27, 309–322.

Lyndon, A.R., Houlihan, D.F., 1998. Gill protein turnover: costs of adaption. Comp. Biochem. Physiol. A 119, 27–34.

Maltby, L., Calow, P., 1989. The application of bioassays in the resolution of environmental problems; past, present and future. Hydrobiologia 188/189, 65–76.

Maltby, L., Naylor, C., 1990. Preliminary observations on the ecological relevance of the *Gammarus* 'scope for growth' assay: effect of zinc on reproduction. Funct. Ecol. 4, 393–397.

McCauley, E., Murdoch, W.W., Nisbet, R.M., Gurney, W.S.C., 1990. The physiological ecology of *Daphnia*: development of a model of growth and reproduction. Ecology 71, 703–715.

Naylor, C., Maltby, L., Calow, P., 1989. Scope for growth in *Gammarus pulex*, a freshwater benthic detritivore. Hydrobiologia 188/189, 517–523.

Rudolph, P., Boje, R., 1986. Ökotoxikologie — Grundlagen für die ökotoxikologische Bewertung nach dem Chemikaliengesetz. Ecomed Fachverlag, Landsberg/Lech.

Sakwinska, O., 1998. Plasticity of *Daphnia magna* life history traits in response to temperature and information about a predator. Freshwater Biol. 39, 681–687.

Stibor, H., 1992. Predator induced life-history shifts in a freshwater cladoceran. Oecologia 92, 162–165.

Stibor, H., Machácek, J., 1998. The influence of fish-exuded chemical signals on the carbon budget of *Daphnia*. Limnol. Oceanogr. 43, 997–1000.

Stuhlbacher, A., Bradley, M.C., Naylor, C., Calow, P., 1992. Induction of cadmium tolerance in two clones of *Daphnia magna* Straus. Comp. Biochem. Physiol. C 101, 571–577.

Tessier, A.J., Henry, L.L., Goulden, C.E., Durand, M.W., 1983. Starvation in *Daphnia*: energy reserves and reproductive allocation. Limnol. Oceanogr. 28, 667–676.

Trubetskova, I., Lampert, W., 1995. Egg size and egg mass of *Daphnia magna*: response to food availability. Hydrobiologia 307, 139–145.

van Leeuwen, C.J., Luttmer, W.J., Griffioen, P.S., 1985. The use of cohorts and populations in chronic toxicity studies with *Daphnia magna*: a cadmium example. Ecotox. Environ. Saf. 9, 26–39.

van Straalen, N.M., Kammenga, J.E., 1998. Assessment of ecotoxicity at the population level using demographic parameters. In: Schüürmann, G., Markert, B. (Eds.), Ecotoxicology. Wiley and Spektrum Akademischer Verlag, New York, pp. 621–644.

Weider, L.J., Pijanowska, J., 1993. Plasticity of *Daphnia* life histories in response to chemical cues from predators. OIKOS 67, 385–392.

Widdows, J., Hawkins, A.J.S., 1989. Partitioning of rate of heat dissipation by *Mytilus edulis* into maintenance, feeding, and growth components. Physiol. Zool. 62, 764–784.

Wilson, R.W., 1996. Physiological and metabolic costs of acclimation to chronic sub-lethal acid and aluminium exposure in rainbow trout. In: Taylor, E.W. (Ed.), Toxicology of Aquatic Pollution. Physiological, Molecular and Cellular Approaches. Society for Experimental Biology, Seminar Series 57. Cambridge University Press, Cambridge, pp. 143–167.

Kapitel VIII

Mixture Toxicity of Priority Pollutants at No Observed Effect Concentrations (NOECs)

Helge Walter, Federica Consolaro, Paola Gramatica, Martin Scholze und Rolf Altenburger
Ecotoxicology, accepted

Mixture Toxicity of Priority Pollutants at No Observed Effect Concentrations (NOECs)

Helge Walter[a], Federica Consolaro[b], Paola Gramatica[b], Martin Scholze[c], Rolf Altenburger[a]*

[a] Department of Chemical Ecotoxicology, UFZ Centre for Environmental Research Leipzig-Halle, Permoserstr. 15 , D-04318 Leipzig, Germany
[b] Institute of Cell Biology, Biochemistry and Biotechnology, University of Bremen, Leobener Str., D-28334 Bremen, Germany.
[c] Department of Structural and Functional Biology, University of Insubria, Via Dunant 3, I-21100 Varese, Italy.

* to whom correspondence should be addressed

ABSTRACT

Environmental exposure situations are often characterised by a multitude of heterogeneous chemicals with ambiguous or unknown modes of action present at low concentrations. While multiple exposure is widely acknowledged, arguments are raised that adverse combined effects might not be evoked by mixtures of substances with dissimilar modes of action and being present at only low concentrations. In this study the combined effect of a multiple mixture composed of structurally dissimilar priority pollutants with mostly unknown modes of action has been investigated using an algal biotest. The concentrations of the components in the mixture equalled statistically estimated, individual no observed effect concentrations (NOECs). The observed mixture toxicity was not only clearly higher than expected for any single substance alone, but also well predictable using the concept of INDEPENDENT ACTION.

Keywords: CONCENTRATON ADDITION, INDEPENDENT ACTION, Mixture toxicity, *Scenedesmus vacuolatus*, priority pollutants, Council Directive 76/464/EEC

INTRODUCTION

Aquatic organisms are typically exposed to multiple mixtures of chemicals, which are heterogeneous in structure as well as in modes of action and which are present at low concentrations. Moreover, for the majority of chemicals information on modes of action is scarce. The predictability of combined effects is however demonstrated only for chemicals, of which the modes of action were known: in particular for chemicals with narcotic (Könemann, 1981a, b, Hermens et al., 1984a, 1985a, b) and specific modes of action (Altenburger et al., 2000, Backhaus et al., 2000, Faust et al., 2000). Risk management procedures for chemicals commonly rely on effect assessments based on single substance evaluations and on the determination of threshold values e.g. no observable effect concentrations (NOECs). Thus the questions arise, whether hazard characterisation focussing on the determination of NOECs for individual substances is sufficient to safeguard against unwanted hazards from mixtures and how to predict combined effects, if the information on the modes of action is scarce or not available.

In the pharmacological and toxicological literature two major concepts - CONCENTRATION ADDITION and INDEPENDENT ACTION (Berenbaum, 1985, Greco et al., 1995, Kortenkamp and Altenburger, 1998) are available to calculate an expected mixture toxicity on the basis of the concentration-response relationships of the individual components. While CONCENTRATION ADDITION is thought to be a valid reference model for mixtures of substances that have similar sites and modes of action, INDEPENDENT ACTION is taken as a reference for chemical mixtures, where the components have different sites and dissimilar modes of action (Pöch 1993, Altenburger et al., 1993). Experimental evidence so far shows, that both concepts indeed show good predictive capabilities for mixtures of specifically acting components, similarly or dissimilarly, respectively (Altenburger et al., 1996, 2000, Backhaus et al., 2000, Faust et al., 2000, Jonker et al., 1996). For the majority of chemicals occurring in the environment, however, we usually do not have sufficient information on modes of action that would allow to classify the chemicals in groups of similar and dissimilar action. In aquatic toxicology, Könemann and Hermens performed pioneering work on mixtures of substances for which information on modes of action is ambiguous or not available. They investigated various mixtures of non-reactive, non-ionized organic chemicals (Könemann, 1981 a, b; Hermens et al., 1984a, 1985a, b), chlorophenols (Könemann and Musch, 1981), chloro- and alkylanilines (Hermens et al., 1984c) and reactive organic halogenated compounds (Hermens et al., 1985c) using mainly fish and daphnid bioassays. Their rational was, that a group of chemicals modelled with a high quality quantitative structure-activity relationship (QSAR), ”may indicate a similar mode of action” of their components (Hermens et al., 1985b). As joint action of mixtures of such compounds they expected concentration addition (Hermens et al., 1984a). Additional studies on mixtures of chemicals with various chemical structures and anticipated different modes of action were performed (Hermens and Leeuwangh, 1982, Hermens et al., 1984b). However, the criteria for the selection of the mixture components was not explicitly stated. In a later study, QSARs were employed to distinguish between groups of compounds of different modes of action (Hermens et al., 1985c). In all the experiments the observed combined effects showed to be concentration additive or partial additive, i.e. slightly less than additive. However, all mixture toxicity results were compared with expectations based on CONCENTRATION ADDITION only and mixture toxicity predictions according to INDEPENDENT ACTION were not calculated. These results were taken as evidence, that CONCENTRATION ADDITION

can be used as model to predict the toxicity of chemical mixtures irrespective of the particular modes of action (van Leeuwen, 1990).

In hazard and risk assessment of mixtures of chemicals at low concentrations the two concepts CONCENTRATION ADDITION and INDEPENDENT ACTION come to almost contradicting conclusions. For CONCENTRATION ADDITION, any chemical present in a mixture, regardless how small its actual concentration is, will contribute to an overall effect with the fraction of the effect concentration of concern. In contrast, the calculation of combined effects according to INDEPENDENT ACTION is based on the components' effects. Consequently, if the components of a mixture are present in concentrations at which they do not show an effect when applied singly, the prediction would be that there will also be no effect of the mixture. These different premises have a direct link to the ongoing debate about the relevance of no observable effect concentrations (NOECs) in risk assessment of chemical mixtures (Chapman et al., 1996, Laskowski, 1995, Moore and Caux, 1997).

For mixtures of similarly acting compounds there seems to be consensus, that thresholds for individual substances are of no relevance for the combined effect of a mixture, whereas mixtures of dissimilarly acting components are often considered to show no combination effect, if the components are present in concentrations below individual thresholds (Cassee et al., 1998, Feron et al., 1998, Henschler at al., 1996, Jonker et al., 1996). Furthermore, mechanistically orientated definitions of similar action, as for example primary interaction of molecules with an identical receptor site, support the view, that the occurrence of similarly acting compounds are rather special cases, whereas dissimilarly acting substances are the general rule (Streffer et al., 2000, Pöch, 1993). Such a view supports the notion that risk assessors may continue to assess chemicals individually (as e.g. EIFAC, 1987) and that threshold values for individual chemicals, which are based on NOECs, represent safe margins also for mixtures of chemicals showing dissimilar action.

The objective of this work was to investigate the occurrence and predictability of the combined effect of a mixture of priority environmental pollutants that approaches a 'real-world situation' (Henschler, 1996), i.e. with dissimilar structures, mostly unknown modes of action and at low concentrations, at which an effect is statistically not observable.

MATERIAL AND METHODS

Selection of mixture components

The selection of mixture components has been realised by structural similarity analysis on 202 priority chemicals derived from EEC list 1 Council directive 76/464 (CSTE/EEC, 1994). The chemical structures of the compounds are described by a set of more than 170 theoretical molecular descriptors giving different structural information. The set of descriptors is constituted by: 38 count descriptors of different kinds of atoms, bonds, functional groups, H-bonds acceptors and donors, 34 topological descriptors (2D-descriptors) including connectivity indices and information indices (Kier et al., 1986; Balaban et al., 1997), 33 non-directional WHIM (Weighted Holistic Invariant Molecular) and 66 directional WHIM descriptors (Todeschini et al., 1994; Todeschini and Gramatica, 1997), encoding 3D structural information. The whole set of descriptors was calculated by the *DRAGON* software (Todeschini and Consonni, 2000a, b) from the

atomic coordinates. These coordinates are obtained from the minimum energy conformations of all the considered compounds by the molecular mechanics method of Allinger (MM+), using the software package *HyperChem* (Rel. 4). More detailed description of the molecular descriptors can be obtained from the Handbook of Molecular Descriptors (Todeschini and Consonni, 2000).

The structural similarity analysis of the compounds, represented by the cited molecular descriptors, was performed by several chemometric methods that allow the grouping of the more structurally similar ones and the highlighting and the final selection of the 11 structurally most dissimilar compounds. Principal Component Analysis (PCA), MultiDimensional Scaling (MDS) and different Hierarchical Cluster Analyses (SCAN and STATISTICA software packages) were the chemometric methods applied for the selection of mixture components, using all the available structural information provided by the molecular descriptors. The chemometric approaches applied here for the selection of the most dissimilar compounds have been already used by Gramatica et al. (2001) in order to verify the structural similarity in sets of triazines and phenylureas.

Test chemicals

All selected compounds were purchased in the highest available purity from Aldrich (Steinheim, FRG), Riedel (Seelze, FRG), Merck (Duesseldorf, FRG), or Sigma (Deisenhofen, FRG). The identity and purity of the compounds are shown in Table 1. Stock solutions of all chemicals were prepared in organic solvents (methanol, p.a., Merck) and stored at –20°C. To prepare test solutions, aliquots were evaporated under N_2 and re-dissolved in algal growth medium overnight.

Tab. 1: Identity, source, charge, and purity of the 11 mixture components used in the combined effect study

EEC NO.[1]	SUBSTANCE	CAS-RN.	LOG K_{ow} [2]	SOURCE	CHARGE	PURITY [%]
130suppl.	Atrazine	1912-24-9	2.61	Riedel-de-Haen	20350	98
11	Biphenyl	92-52-4	3.98	Merck	S 16211810	99
14	Chloral hydrate	302-17-0	0.99	Merck	K 24366421	99
122	2.4.5-Trichlorophenol	95-95-4	3.70	Riedel-de-Haen	30250	99
[99]	Fluoranthene	206-44-0	5.20	Aldrich	12301-058	98
85	Lindane	58-89-9	4.14	Dr. Ehrensdorfer	50621	99
96	Naphthalene	91-20-3	3.30	Aldrich	0309-038	99
100	Parathion	56-38-2	3.83	Dr. Ehrensdorfer	60729	99
103	Phoxim	14816-18-3	4.39	Riedel-de-Haen	62700	99
115	Tributyltin chloride	1461-22-9	5.80	Riedel-de-Haen	50230	96
126	Triphenyltin chloride	639-58-7	2.83	Merck	50886234	98

[1] according to CSTE/EEC, 1994
[2] UBA. 1999

Test organisms and culture conditions

Liquid cultures of the unicellular green alga *Scenedesmus vacuolatus* Shih. et Krauss, strain 211-15, culture collection Pringsheim (SAG Göttingen, Germany) were grown photoautotrophically at 28 ± 0.5°C in an inorganic, sterilised medium adjusted to pH 6,4 under conditions specified earlier (Altenburger et al., 1990). Cells were synchronised by light:dark changes of 14:10 h and a periodic dilution to a standard cell density of 10^6

cells/mL before the onset of the light phase of the growth cycle (t_0). Synchronisation was checked by analysis of the cell size distribution at t_0.

Determination of concentration-response relationships
Concentration-response relationships of the test compounds were experimentally determined using a 24 h test under synchronised conditions taking the inhibition of algal cell reproduction as effect parameter. The initial cell density was set to $7.5*10^4$ cells/mL. Gas tight test tubes (PYREX 15, QVF, Wiesbaden, Germany) were used as test vessels. Culture volumes were 8 ml with a headspace of 2 ml. The test medium was the same as for cultivation but enriched with 1.9 mmol/L $NaHCO_3$ providing a final pH of the medium of 6.9 ± 0.2. Illumination was ensured by a combination of two types of fluorescent light tube (L36W/41 Interna, L36W/11 daylight, Osram, Berlin, Germany) with an intensity of 13-18 W/m² (22-33 kLux) providing a photosynthetic active radiation of 350 µE s^{-1} m^{-2} at the surface of the test vessels. The aqueous test substances were added to the cultures at t_0. The experimental design is characterised by 12 test concentrations as triplicates covering 1 to 80% effect, 6 untreated cultures as control values and a geometrical dilution series adjusted to the steepness of the concentration-response relationship, which were previously determined in separately conducted range-finding experiments. The data obtained in independently conducted experiments was pooled for data analysis. Aliquot samples of the cultures were taken in duplicates at t_0 and at the end of the standard algal reproduction cycle (t_{24}) and the mean cell number was analysed twice using a CASY II-particle counter (Schärfe System, Reutlingen, Germany). The inhibition of cell reproduction was calculated by normalising the data to the results of control cultures.

Chemical analysis
In order to check whether the concentrations of the test chemicals were achieved in the applied stock solutions as well as in the test vessels, HPLC- and GC-analyses were performed, typically using rp-18 endcapped columns, with mobile phases such as acetonitrile/water or methanol/water and a compound specific UV detection wavelength (between 220 and 440 nm). Provided test concentrations of the substances are therefore analytically corrected values with the exception of chloral hydrate. Verification of substance stability over the exposure time was conducted for all substances either by analytical determination of concentrations under test conditions after 24 h or by reproducing the observed concentration effect data with identical dilution series using stock solutions pre-incubated under test conditions for 24 h.

Data analysis
The concentration-response relationships of the single substances were biometrically modelled by using a ‘best-fit’ approach (Scholze et al., 2001). For that purpose, ten different 2- or 3-parametric sigmoidal regression models – including the commonly used Probit-, Logit and Weibull-models – were fitted to every experimental data set. The parameters of the models were estimated using a generalised least-square approach. For each individual set of data the best fitting model was chosen on the basis of statistical criteria. The statistical uncertainty of the EC_{50} was estimated using the bootstrap approach (Scholze et al., 2001). The NOEC was determined using the Dunnett test (1964). The toxicity of the single compounds was evaluated to correspond with a toxicity that is based on the substances hydrophobicity only (Lipnick, 1995). For that purpose, for each component a baseline toxicity was calculated using a QSAR based on toxicity data from 6 alcohols (Walter, 2000).

Prediction of mixture toxicity

As the mixture is composed of components at their individual NOECs, the concentration of each component in the mixture can be expressed as fraction of the total mixture concentration. Consequently, a total concentration of the mixture, at which a certain effect is generated, can be calculated by CONCENTRATION ADDITION according to:

$$ECx_{Mix} = (\sum_{i=1}^{n} \frac{p_i}{ECx_i})^{-1} \qquad \text{[Eq. 1]}$$

where ECx_{Mix} is the total concentration of the mixture provoking x % effect, ECx_i the concentration of component i also provoking x% effect, but if applied singly, and p_i denotes the fraction of component i in the mixture.

The calculation of total mixture concentrations for various effect levels lead to a complete concentration-effect relationship to be expected, given the components act concentration additivly. It furthermore allows to calculate the expected effect for the total concentration of the mixture, at which the components are present in their individual NOECs, which is evidently more than 99,9%.

On the basis of the determined NOECs of the individual substances, the expected mixture effect according to INDEPENDENT ACTION can be calculated according to eq. [2]. The predicted mixture effect according to INDEPENDENT ACTION ($E(NOEC_1 + ... + NOEC_n)_{Mix}$) was derived using the calculated effects of the mixture components (using the appropriate concentration-response functions) if applied singly at their no observed effect concentrations ($E(NOEC_i)$) as follows:

$$E\,(NOEC_1 + ... + NOEC_n)_{Mix} = 1-\prod_{i=1}^{n} (1-E(NOEC_i)) \qquad \text{[Eq. 2]}$$

RESULTS

Single substance toxicity

An example of the quality of experimental concentration-response data and the corresponding regression fit is given in figure 1 for the algal toxicity of naphthalene. The statistical uncertainty of the EC50 is smaller than 9% of this effect concentration (mean of 13% for all components). The results of the concentration-response

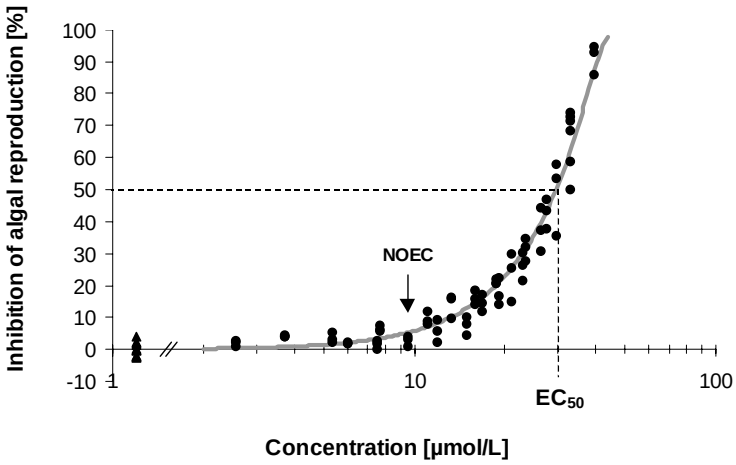


Fig .1: Concentration-response data (●) and concentration-response function for the inhibition of reproduction of *Scenedesmus vacuolatus* by naphthalene. Control values are also denoted (▲).

analysis of 11 aquatic priority pollutants, selected from EEC list 1 (Council directive 76/464), are provided in Table 2. The observed algal toxicity of the single substances span more than 5 orders of magnitude stretching from an EC_{50} of about 1700 µmol/L for the least toxic chemical chloral hydrate to an EC_{50} value of 0.082 µmol/L for the known specific photosystem II inhibitor atrazine.

Tab. 2: Single substance toxicity of the mixture components to the green algae *Scenedesmus vacuolatus*. θ_i denotes the parameter of the concentration-response function; the 95% confidence interval is given in brackets.

SUBSTANCE	N[1]	N[2]	REGRESSION-MODEL[3]	θ_1	θ_2	θ_3	MAXIMAL TESTCONC.[4] [µmol/L]	MINIMAL DILUTION-FACTOR[4]	NOEC[5] [µmol/L]	EC_{50} [µmol/L]	EC_{50} BASELINE[6] [µmol/L]
Atrazine	36	6	BCP	2.461	0.866	-0.0987	0.28	1.40	0.026	0.082 [0.075 - 0.089]	1100.0
Biphenyl	36	5	GL	-7.032	23.194	0.2346	3.60	1.34	0.62	1.50 [1.38 - 1.64]	78.0
Chloral hydrate	36	7	BCP	-26.67	7.304	-0.221	2600.0	1.26	650.0	1700.0 [1624 - 1803]	21000.0
2,4,5-Trichlorophenol	72	12	L	-0.802	15.826		1.10	1.05	0.80	1.10 [1.06 - 1.24]	140.0
Fluoranthene	34	6	GL	82.844	94.777	0.0606	0.13	1.15	0.063	0.180 [0.098 - 0.106]	8.0
Lindane	36	5	BCP	-2.027	0.817	0.5584	5.90	1.19	1.80	4.80 [4.60 - 4.90]	64.0
Naphthalene	72	11	GL	-85.44	52.673	0.087	40.00	1.20	9.50	30.00 [28.44 - 31.05]	290.0
Parathion	35	6	W	-8.001	6.071		20.00	1.14	9.90	18.00 [17.18 - 19.20]	110.0
Phoxim	36	6	W	-0.091	1.805		2.80	2.10	0.015	0.700 [0.637 - 0.784]	37.0
Tributylttin chloride	36	6	GL	30.977	82.956	0.0438	0.40	1.30	0.113	0.270 [0.256 - 0.289]	2.7
Triphenyltin chloride	35	6	W	2.194	4.789		0.57	1.30	0.118	0.290 [0.265 - 0.321]	730.0

[1] number of data points
[2] number of controls

[3] BCP = Box-Cox-Probit :

GL = Generalized Logit : $$\text{Effect} = \frac{1}{2\pi}\int_{-\infty}^{k(\text{conc.})}\exp(-u^2/2)du, k(\text{Conc.}) = \theta_1 + \theta_2\frac{\text{Conc.}^{\theta_3}-1}{\theta_3} = \text{Probit}\left(\theta_1 + \theta_2\frac{\text{Conc.}^{\theta_3}-1}{\theta_3}\right)$$

L = Logit : $$\text{Effect} = \frac{1}{(1+\exp(-\theta_1 - \theta_2\log_{10}(\text{Conc.})))^{\theta_3}}$$

W = Weibull : $$\text{Effect} = \frac{1}{(1+\exp(-\theta_1 - \theta_2\log_{10}(\text{Conc.})))}$$

$$\text{Effect} = 1-\exp(-\exp(\theta_1 + \theta_2\log_{10}(\text{Conc.})))$$

[4] values differ for different experiments per substance
[5] determined with the DUNETT test
[6] log EC_{50} (mol/L) = -0,81 * log *K*ow -0,87 (baseline toxicity according to Walter, 2000)

Mixture toxicity

A mixture was composed containing all eleven substances in their individual NOECs and a pH adjusted to 7.5. This was necessary as preliminary modelling of the pH-dependent algal toxicity of 2,4,5-trichlorophenol indicated (data not shown; Walter, 2000), that the fraction of unionised 2,4,5-trichlorophenol (pK_a 6.9) could have an impact on the observable mixture toxicity, while under basic conditions the ionised and least toxic

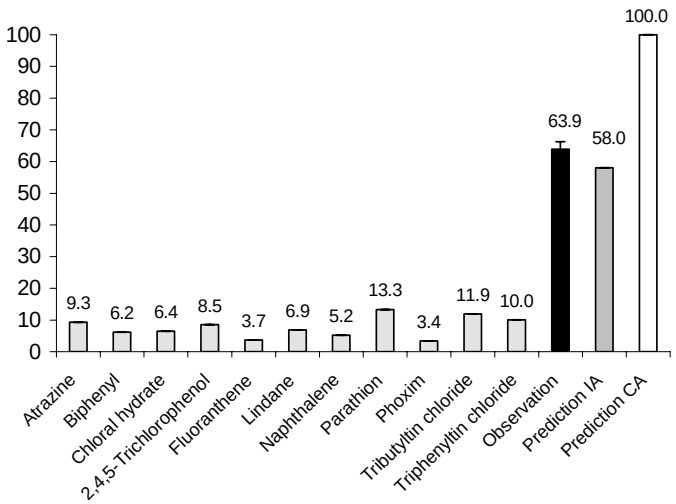


Fig. 2: Predicted and observed inhibition of reproduction of *Scenedesmus vacuolatus* for a mixture of 11 aquatic priority pollutants present at individual no observed effect concentrations.
IA - concept of INDEPENDENT ACTION; CA - concept of CONCENTRATION ADDITION.

form would prevail (Könemann and Musch, 1981, Schüürmann, 1998). This mixture was than tested in 18 replicates using the same one-generation algal reproduction test as employed for the description of the single substances. Figure 2 shows expected and observed algal toxicity. On the left hand side the effects of all compounds at NOEC concentrations are depicted as estimated from the concentration response functions given in Table 2. On the right hand side of Figure 2, the predicted mixture toxicity calculated from the eq. [1] (CONCENTRATION ADDITION) and eq. [2] (INDEPENDENT ACTION) as well as the experimentally observed combined effect are shown. The magnitude of the combined effect is significantly higher than the effect elicited of any component at its individual NOEC. Obviously, the effect prediction based on CONCENTRATION ADDITION overestimates the observed effect of the mixture, while the expected effect of the mixture according to INDEPENDENT ACTION (58%) provides a rather good estimate of the experimentally determined average mixture toxicity of about 64 % ± 3 %.

DISCUSSION

Single substance toxicity

A comparison of the EC_{50} values, derived from the concentration-response functions for the inhibition of algal reproduction after one generation of exposure to the single substances with literature data is displayed in Table 3. For chloral hydrate and phoxim no algal toxicity data were found. The other compounds show reasonable agreement of the experimental findings with literature data, except for fluoranthene and the organotin compounds. For fluoranthene the algal toxicity reported here is about two orders of magnitude higher than any previously reported. This might be due to photomodification products given the relatively high light conditions used in this test as compared to standard ISO protocols. The organotin algal toxicity reported in the literature seems strikingly higher than observed in this study. This might be attributed to differences in exposure time or regime – compared are several days of exposure against 24h used in our biotest. Although the deviating toxicity observed for fluoranthene and the organotin compounds compared to literature values cannot be comprehensively explained, the reproducibility of the concentration-response data gained in independent experiments is rather good. In conclusion, there is no indication of undue technical or biological variability which could confound the assessment of the mixture toxicity prediction.

Mixture toxicity

The results from the study of a mixture of 11 structurally dissimilar aquatic priority pollutants with mostly unknown modes of action at concentrations that equal individual no effect concentrations, show (i) that a combined effect is observable, which shows clearly a higher intensity than that of any individual compound, and (ii) that the magnitude of this effect is more precisely predicted by the model of INDEPENDENT ACTION than by CONCENTRATON ADDITION.

The former result (i) seems in contradiction with earlier findings for multiple mixtures of chemicals at no observed adverse effect level (NOAEL) or lowest observed adverse effect level (LOAEL) concentrations in rats (Cassee et al., 1998). Furthermore, Hermens et al. (1984b) and Hermens and Leeuwangh (1982) reported, that the observed toxicity of a mixture constituted of components with "various structures and probable modes of action" in daphnids was predictable with the concept of CONCENTRATION ADDITION, which is in opposition to the findings (ii) in our study.

Tab. 3: Comparison of observed algal toxicity to the green algae *Scenedesmus vacuolatus* and effects to algae reported in the literature.

NAME	CAS RN.	EC_{50} OBSERVED mg/L	EC_{50} LITERATURE mg/L	EFFECT PARAMETER & EXPOSURE DURATION	SPECIES	REFERENCE
Atrazine	1912-24-9	0,018	0,02-0,11	growth, 4 d	*Scenedesmus subspicatus*	Grimme et al., 1994
Biphenyl *	92-52-4	0,230	5	growth 3 d	Chlorella pyrenoidosa	Ramos et al., 1999
Chloral hydrate	302-17-0	280,000	no data found			
2,4,5-Trichlorophenol	95-95-4	0,220	0,39-3,1	growth 4 d	Selenastrum capricornutum	US-EPA, 1978
Fluoranthene	206-44-0	0,036	4,4-64,3	growth 4 d	*Selenastrum capricornutum*	US-EPA, 1978
Lindane	58-89-9	1,400	3,2	growth 3 d	*Scenedesmus subspicatus*	Schäfer et al., 1994
			1,3-3,9	growth 3-10 d	various green algae	Geyer et al., 1985
Naphthalene	91-20-3	3,800	3	physiol. endpoint 0.17 d	Selenastrum capricornutum	Millemann et al., 1984
			33	growth 1 d	*Chlorella vulgaris*	Kauss and Hutchinson, 1975
Parathion	56-38-2	5,300	0,008-25	population parameter	blue-green and green algae	Cole et al., 1974; Mostafa et al.. 1991
Phoxim	14816-18-3	0,210	no data found			
Tributyltin chloride	1461-22-9	0,163	0,0034	growth 4d	*Scenedesmus acutus*	Huang et al., 1993
			0,044	growth 4d	*Chlorella vulgaris*	Huang et al., 1993
Triphenyltin chloride	639-58-7	0,113	0,0009	growth 12 d	*Scenedesmus quadricauda*	Fargasova, 1997
			0,0033	physiol. endpoint 2 d	*Scenedesmus quadricauda*	Fargasova, 1997
			0,0056	growth 4 d	*Scenedesmus acutus*	Huang et al., 1993

* literature data for 1,1-biphenyl-2-ol

The first aspect to look at is the compound selection in relation to expectable modes of action. The choice of mixture compounds was made on the basis of chemometric similarity analyses using only structural information encoded in the different kinds of molecular descriptors without any link to chemical activity or biological response. Thus, it cannot be excluded that at least some of the selected compounds have common biological potencies, like for example the organotin compounds TBTC and TPTC, which are both known inhibitors of photosynthesis (Fargasova, 1998). But it is also clear, that except for atrazine, which is a specific photosystem II inhibitor, and TBTC and TPTC, which specifically inhibit oxidative phosphorilation and photophosphorilation , all other compounds do not have a known specific mechanism of action in algae. However, none of the compounds evoke a toxicity, which is low enough to be described on the basis of a narcotic mode of action solely (Lipnick, 1995). This is demonstrated by the calculated baseline toxicity of all substances (Table 2), which exceed their EC_{50} by a factor ranging from 6 to 13.415. Thus, narcosis as similar and hence common mechanism of action of all chemicals, can be excluded.

The dissimilarity of modes of action of the mixture components obviously seems sufficient to predict the toxicity of the mixture more precisely with the model of Independent Action than with the model of CONCENTRATION ADDITION. However, since the molecular modes of action of the components are mostly unknown, the possibility, that their modes of action are similar cannot be ruled out. To reduce the probability of false application of Independent Action, additional studies with the same mixture on the

stability of the mixture toxicity results were performed. Ten additional mixture experiments were conducted, each time leaving out one or two mixture components with presumed potential to alter the observed mixture toxicity (e. g. the substances with a known specific mode of action in algae, atrazine, TBTC, TPTC) (data not shown; Walter, 2000). All these experiments showed a predictability of the mixture toxicity, which was comparable to the predictability of the eleven component mixture. These findings strongly indicate, that the modes of action of mixture components are indeed dissimilar in a way, that is sufficient to predict the mixture toxicity with the concept of Independent Action.

These results seem to be in contradiction to findings of Hermens et al. (1984b) and Hermens and Leeuwangh (1982), who reported, that the toxicity of mixtures of substances "with various structures and probable modes of action" was predictable with the concept of CONCENTRATION ADDITION. In these studies, however, no explicit prediction according to the concept of Independent Action was calculated. Nor was it demonstrated, that the mixture components had a toxicity higher than to be explained by their lipophilicity only, which does not exclude narcosis as common mode of action. Even if it was shown, that the mixture components had a more specific mode of action (Hermens and Leeuwangh, 1982), the observed toxicity of the investigated mixture was assessed to be predictable with CONCENTRATION ADDITION. Although we have put some effort into selecting substances with dissimilar structures by chemometrical means, it is not possible to conclude from the results in our study, that structural dissimilarity is strictly linked to dissimilarity in modes of action, as it is unknown which chemical descriptors describe what chemical and biological interaction.

It has repeatedly been proposed, that Independent Action might be a concept of good predictive capability for mixtures of dissimilarly acting compounds present at high concentrations (Broderius et al., 1995, Henschler et al., 1996, Cassee et al., 1998, Pape-Lyndstrom and Lydy, 1997). Originally, the concept of INDEPENDENT ACTION goes back to the idea of statistical independence of effects. Later, it was translated into pharmacological terms regarding the sites and modes of action of chemicals. However, if dissimilarly acting components of a mixture are present at concentrations as low as their individual NOEC, and provided that this concentration produces no toxic effect, then there should be no toxicity of the mixture also, unless there is synergistic interaction (Cassee et al., 1998, Henschler at al., 1996). Implicit, this is also the widespread assumption in the field of chemical regulation, whenever decisions on risks of chemicals are based on information about individual compounds only (explicitly e.g. EIFAC, 1987). Experimental evidence in this area is scarce. Cassee et al. (1998) summarised a series of studies that used a 4-week toxicity biotest with rats to investigate the combined effects of 4-9 compound mixtures at no observed adverse effect level (NOAEL) or lowest observed adverse effect level (LOAEL) concentrations on various toxicity parameters. For mixtures of chemicals with different target organs and/or different modes of action, they suggest from their findings ”that exposure to the combinations of chemicals did not constitute an evidently increased hazard, provided the dose level of each individual chemical is a NOAEL.” There might be two aspects to consider when comparing these findings with the results of this study.

First of all, the data for rats were produced utilising an acute toxicity test while the algal data presented here represent in fact a chronic one-generation biotest. This gives reason for concern that exposure time might be of major concern for risk statements. The influence of exposure time on the magnitude of observable mixture effects has

been investigated by Altenburger and Backhaus (2000) who reported, that the predictability of the toxicity of binary mixtures of dissimilarly acting industrial chemicals is improved with extended exposure time in bacteria.

Secondly, when using the model of INDEPENDENT ACTION for calculating expected combined effects of a multiple mixture of components at low concentrations, it is necessary to reliably estimate rather small effects of the single components. This can be in contrast to the usage of CONCENTRATION ADDITION, which requires estimation of effect concentrations. E.g. the prediction of an evidently severe mixture toxicity of say 50% for a 10 compound mixture requires reliable effect estimations in the region of 5% for each individual chemical. But, to calculate the expected mixture concentration producing an effect of 50% according to CONCENTRATION ADDITION, only the information about the EC50 of every compound is needed. This may be the reason why combined effect predictions at low effect levels based on INDEPENDENT ACTION are rarely found in the literature. In their study, Cassee et al. (1998) do not provide any information on the effect estimation procedure applied nor do they demonstrate the quality of the dose response data for the single components. In our study, the estimated effects, which correspond to the individual NOECs of the mixture components, range from 3,7% to 13,3% (Figure 2). Equivalent values derived from standard toxicity tests were reported to range from 10-30% (Moore and Caux, 1997). Considering, that NOECs and their corresponding effects depend strongly on the experimental design as well as on the biological variability of the test system used, the relatively low effect values in this study give an indication of the quality of the data sets produced. In this study, not only an experimental design was used allowing to achieve this quality of data, but also specific efforts were made to model the concentration-response relationships by biometrical means (Grimme et al., 1998, Scholze et al., 2001). This might be of special importance, when the effects, generated at a NOEC of the mixture components, are estimated on the basis of modelled concentration-response relationships and when estimated low effects are used to calculate the toxicity of the mixture according to the model of INDEPENDENT ACTION.

CONCLUSIONS

The presented results of this study allow to conclude that no observed effect concentrations (NOECs) for individual compounds are no safeguard against unwanted toxicity from mixtures approximating an exposure situation likely to occur in the environment.
Predicting and assessing the toxicity of mixtures of structurally dissimilar chemicals with ambiguous or unknown modes of action, independent joint action of the components must be considered as possible combined action. Whether independent combined action of structurally dissimilar chemicals is of general importance should be further investigated.

ACKNOWLEDGEMENTS

This work was supported by the Environment & Climate Programme of the European Commission, Contract N. ENV4-CT96-0319; we gratefully acknowledge the co-ordinator of this project Prof. L H Grimme.

REFERENCES

Altenburger R, Backhaus T, 2000. Der Faktor Zeit bei der Beurteilung von biologischen Wirkungen. In: *Biotests in der Praxis*, Mücke W. (ed), Institut für Toxikologie und Umwelthygiene, Technische Universität München., pp. 61-74.

Altenburger R, Bödeker W, Faust M, Grimme LH, 1990. Evaluation of the isobologram method for the assessment of mixtures of chemicals. Combination effect studies with pesticides in algal biotests. Ecotoxicology and Environmental Safety 20: 98-114.

Altenburger R, Bödeker W, Faust M, Grimme LH, 1993. Aquatic Toxicology, Analysis of combination effects. In: Handbook of hazardous materials, Corn M (Ed), New York, Academic Press.

Altenburger R, Bödeker W, Faust M, Grimme LH, 1996. Regulations for combined effects of pollutants: Consequences from risk assessment in aquatic toxicology. Food and Chemical Toxicology 34: 1155-1157.

Altenburger R, Backhaus T, Boedeker W, Faust M, Scholze M. ,Grimme LH, 2000. Predictability of the toxicity of multiple chemical mixtures to *Vibrio fisheri*: mixtures composed of similarly acting chemicals. Environmental Toxicology and Chemistry 19: 2341-2347.

Backhaus T, Altenburger R, Boedeker W, Faust M, Scholze M. ,Grimme LH, 2000. Predictability of the toxicity of a multiple mixture of dissimilarly acting chemicals to *Vibrio fischeri*. Environmental Toxicology and Chemistry 19: 2348-2356.

Balaban AT, 1997. From chemical topology to three-dimensional geometry. New York, Plenum Press.

Berenbaum MC, 1985. The expected effect of a combination of agents: the general solution. Journal of Theoretical Biology 114:413-431.

Broderius SJ, Kahl MD, Hoglund MD, 1995. Use of joint toxic response to define the primary mode of toxic actionn for diverse industrial organic chemicals. Environmental Toxicology and Chemistry 14: 1591-1605.

Cassee FR, Groten JP, van Bladeren PJ, Feron VJ, 1998. Toxicological evaluation and risk assessment of chemical mixtures. Critical Reviews in Toxicology 28: 73-101.

Chapman PM, Cadwell RS, Chapman PF, 1996. A warning: NOECs are inappropriate for regulatory use. Environmental Toxicology and Chemistry 15: 77-79.

Cole DR, Plapp, Jr. FW, 1974. Inhibition of growth and photosynthesis in *Chlorella pyrenoidosa* by a polychlorinated biphenyl and several insecticides. Environmental Entomology 3(2): 217-220.

CSTE/EEC (1994). Scientific Advisory Committee Activity Report published by the Commission of the European Communities, Office for Official Publication of the E.C., Luxembourg: EUR (1992-1993).

Dunnett CW, 1964. New tables for multiple comparisons with a control. Biometrics 20: 482-491.

European Inland Fisheries Advisory Commission (EIFAC), 1987. Water quality criteria for European freshwater fish. Revised report on combined effects on freshwater fish and other aquatic life of mixtures of toxicants in water. EIFAC Technical Paper No. 37, Rev. 1.

Fargasova A, 1997. Comparative study of ecotoxicological effect of triorganotin compounds on various biological subjects. Ecotoxicology and Environmental Safety 36: 38-42.

Fargasova A, 1998. Comparison of effects of tibutyl-, triphenyl-, and tribenzyltin compounds on freshwater benthos and algae *Scenedesmus quadricauda*. Bulletin of Environmental Contamination and Toxicology 60: 9-15.

Faust M, Altenburger R, Backhaus T, Boedeker W, Scholze M, Grimme LH, 2000. Predictive assessment of the aquatic toxicity of multiple chemical mixtures. Journal of Environmental Quality 29: 1063-1068.

Feron VJ, Cassee FR, Groten JP, 1998. Toxicology of chemical mixtures: international perspective. Environmental Health Perspectives 106, Supplement 6: 1281-1289.

Geyer H, Scheunert I, Korte F, 1985. The effects of organic environmental chemicals on the growth of the alga *Scenedesmus subspicatus*: a contribution to environmental biology. Chemosphere 14(9): 1355-1369.

Gramatica P, Vighi M, Consolaro F, Todeschini R, Finizio A, Faust M, 2001. QSAR approach for the selection of congeneric compounds with a similar toxicological mode of action. Chemosphere 42: 873-883.

Greco WR, Bravo G, Parsons JC, 1995. The search for synergy: a critical review from a response surface perspective. Pharmacological Reviews 47: 331-385.

Grimme LH, Altenburger R, Bödeker W, Faust M, 1994. Kombinationswirkungen von Schadstoffen – Toxizität binärer Kombinationen von Pestiziden und Tensiden im Algenbiotest. Forschungsbericht 102 07 205, Umweltforschungsplan des Bundesministers für Umwelt, Naturschutz und Reaktorsicherheit.

Grimme LH, Altenburger R, Backhaus T, Boedeker W, Faust M, Scholze M, 1998. Predictability and assessment of the aquatic toxicity of mixtures of substances – multi-component mixtures of dissimilarly acting chemicals at low effect concentrations (in German). UFZ-Bericht Nr. 25/1998, Leipzig, Germany, Umweltforschungszentrum Leipzig-Halle GmbH.

Henschler D, Bolt HM, Jonker D, Pieters MN, Groten JP, 1996. Experimental designs and risk assessment in combination toxicology: panel discussion. Food and Chemical Toxicology 34: 1183-1185.

Hermens J, Broekhuysen E, Canton H, Wegman R, 1985a. Quantitative structure activity relationships and mixture toxicity studies of alcohols and chlorohydrocarbons: effects on growth of *Daphnia magna*. Aquatic Toxicology 6: 209-217.

Hermens J, Busser F, Leeuwangh P, Musch A, 1985b. Quantitative structure-activity relationships and mixture toxicity of organic chemicals in *Photobacterium phosphoreum*: the MICROTOX test. Ecotoxicology and Environmental Safety 9: 17-25.

Hermens J, Canton H, Janssen P, Jong R, 1984a. Quantitative structure-activity relationships and toxicity studies of mixtures of chemicals with anaesthetic potency: acute lethal and sublethal toxicity to *Daphnia magna*. Aquatic Toxicology 5: 143-154.

Hermens J, Canton H, Steyger N, Wegmann R, 1984b. Joint effects of a mixture of 14 chemicals on mortality and inhibition of reproduction of *Daphnia magna*. Aquatic Toxicology 5: 315-322.

Hermens J, Leeuwangh P, 1982. Joint toxicity of mixtures of 8 and 24 chemicals to the guppy (*Poecilia reticulata*). Ecotoxicology and Environmental Safety 6: 302-310.

Hermens J, Leeuwangh P, Musch A, 1984c. Quantitative structure-activity relationships and mixture toxicity studies of chloro- and alkylanilines at an acute lethal toxicity level to the guppy (*Poecilia reticulata*). Ecotoxicology and Environmental Safety 8: 388-394.

Hermens J, Leeuwangh P, Musch A, 1985c. Joint toxicity of mixtures of groups of organic aquatic pollutants to the guppy (*Poecilia reticulata*). Ecotoxicology and Environmental Safety. 9: 321-326.

Huang G, Bai Z, Dai S, Xie Q, 1993. Accumulation and toxic effect of organometallic compounds on algae. Applied Organometallic Chemistry 7(6): 373-380.

HyperChem Rel.4 for Windows 95 Autodesk Inc. Sausalito, CA.

Jonker D, Wouterson RA, Feron VJ, 1996. Toxicity of mixtures of nephrotoxicants with similar or disssimilar mode of action. Food and Chemical Toxicology 34: 1075-1082.

Kauss PB, Hutchinson TC, 1975. The effects of water-soluble petroleum components on the growth of *Chlorella vulgaris beijerinck*. Environmental Pollution 9(3): 157-174.

Kier LB, Hall LH, 1986. Molecular connectivity in structure-activity analysis. UK, Research Studies Press Letchworth.

Könemann H, 1981a. Fish toxicity tests with mixtures of more than two chemicals: a proposal for a quantitative approach and experimental results. Toxicology 19: 229-238.

Könemann H, 1981b. Quantitative-structure-activity relationships in fish toxicity studies. 1. Relationships for 50 industrial pollutants. Toxicology 19: 209-221.

Könemann H, Musch A, 1981. Quantitative structure-activity relationships in fish toxicity studies. Part 2: The iunfluence of pH on the QSAR of chlorophenols. Toxicology 19: 223-228.

Kortenkamp A, Altenburger R, 1998. Synergisms with mixtures of xenoestrogens - a reevaluation using the method of isoboles. Science of the total Environment 221: 59-73.

Laskowski R, 1995. Some good reasons to ban the use of NOEC, LOEC and related concepts in ecotoxicology. Oikos 73: 140-143.

Lipnick RL (1995) Structure-activity relationships. In: Fundamentals of aquatic toxicology. 2nd edition, Rand GR (ed), London, UK, Taylor & Francis.

Leeuwen van, K. 1990. Ecotoxicological effect assessment in the netherlands: recent developments. Environmental Management 14: 779-792.

Millemann RE, Birge WJ, Black JA, Cushman RM, Daniels KL, Franco PJ, Giddings JM, 1984. Comparative acute toxicity to aquatic organisms of components of coal-derived synthetic fuels. Transactions of the American Fisheries Society 113(1):7 4-85.

Moore DRJ, Caux P-Y, 1997. Estimating low toxc effects. Environmental Toxicology and Chemistry 16: 794-801.

Mostafa IY, Shabana EF, Khalil Z, Mostafa FIY, 1991. The metabolic fate of 14c-parathion by some fresh water phytoplankton and its possible effects on the algal metabolism. Journal of Environmental Science and Health, Part B—Pesticides, Food Contaminants, and Agricultural Wastes 26(5/6): 499-512.

Pape-Lyndstrom PA, Lydy MJ, 1997. Synergistic toxicity of atrazine and organophosphate insecticides contravenes the response addition mixture model. Environmental Toxicology and Chemistry 11: 2415-2420.

Pöch G, 1993. Combined effects of drugs and toxic agents. Modern evaluation in theory and practice. Wien, New York, Springer Verlag.

Ramos EU, Vaes WHJ, Mayer P, Hermens JLM, 1999. Algal growth inhibition of *Chlorella pyrenoidosa* by polar narcotic pollutants: toxic cell concentrations and QSAR modelling. Aquatic Toxicology 46 (1): 1-10.

SCAN - Software for Chemometric Analysis (1995). rel. 1.1 for Windows, , Minitab (USA).

Schäfer H, Hettler H, Fritsche U, Pitzen G, Roderer G, Wenzel A, 1994. Biotests using unicellular algae and ciliates for prediciting long-term effects of toxicants. Ecotoxicology and Environmental Safety 27(1): 64-81.

Scholze M, Boedeker W, Faust M, Backhaus T, Altenburger R, Grimme LH, 2001. A general best-fit method for concentration-response curves and the estimation of low-effect concentrations. Environmental Toxicology and Chemistry 20 (2): 448-457.

Schüürmann G, 1998. Ecotoxic modes of action of chemical substances. In: Ecotoxicology. Schüürmann G, Markert B (eds), New York, John Wiley and Spektrum Akademischer Verlag.

Streffer C, Bücker J, Cansier A, Cansier D, Gethmann CF, Guderian R, Hanekamp G, Henschler D, Pöch G, Rehbinder E, Renn O, Slesina M, Wuttke K, 2000. Umweltstandards. Kombinierte Expositionen und ihre Auswirkungen auf den Menschen und seine Umwelt. Europäische Akademie zur Erfoschung von Folgen wissenschaftlich-technischer Entwicklungen Bad Neuenahr-Ahrweiler GmbH, Bd. 5, Berlin, Springer.

Todeschini R, Consonni V, 2000a. DRAGON – Software for the calculation of molecular descriptors, version 1.0 for Windows. Free download from http://www.disat.unimib.it/chm.

Todeschini R, Consonni V, 2000b. Handbook of Molecular Descriptors, Weinheim, Germany, Wiley-VCH.

Todeschini R, Gramatica P, 1997. 3-D modelling and prediction by WHIM descriptors. Part 5. Theory development and chemical meaning of the WHIM descriptors. Quantitative Structure-Activity Relationships 16: 120-125.

Todeschini R, Lasagni M, Marengo E, 1994. New molecular descriptors for 2D and 3D structures theory. Journal of Chemometrics 8: 263-272.

UBA (Umweltbundesamt), 1999. Dokumentation wassergefährdender Stoffe – Datenblattsammlung: Grundwerk mit 3. Ergänzungslieferung, Stand: Juni 1998. Stuttgart, Hirzel.

U.S.-EPA (Environmental Protection Agency), 1978. In-depth studies on health and environmental impacts of selected water pollutants. Contract No. 68-01-4646, Duluth, U.S. EPA.

Walter H, 2000. Kombinationswirkungen von Umweltchemikalien – Zur Analyse der milieuabhängigen Mischungstoxizität von Kontaminanten mit unbekanntem Wirkungsmechanismus in umweltrelevanten Konzentrationen. Dissertation. Universität Halle-Wittenberg.

Kapitel IX

Erfolgskontrolle von Grundwasser-Sanierungen: Wird die Toxizität durch Behandlung reduziert?

Rolf Altenburger, Eberhard Küster, Falk Dorusch, Bettina Meißner, Holger Weiß und Gerrit Schüürmann
Grundwasser, akzeptiert

Erfolgskontrolle von Grundwasser- Sanierungen: Wird die Toxizität durch Behandlung reduziert ?

Rolf Altenburger[1], Eberhard Küster[1], Falk Dorusch[1], Bettina Meißner[1], Holger Weiss[2], Gerrit Schüürmann[1]

UFZ- Umweltforschungszentrum Leipzig-Halle GmbH
[1] Sektion Chemische Ökotoxikologie
[2] Projektbereich Industrie- und Bergbaufolgelandschaften
Permoserstr. 15, 04318 Leipzig

Kurzfassung

Im folgenden Artikel wird der Einsatz kontinuierlicher on line Biotests zur Sanierungsüberwachung von Grundwasser beschrieben. Zur Einordnung der Befunde hinsichtlich der erfaßten Effektqualitäten werden Ergebnisse verschiedener diskontinuierlicher Biotests wie des Leuchtbakterien-, Algen-, Fischei-, Daphnien- und bakteriellen Genotoxizitätstest beschrieben. Es zeigte sich, daß der automatisierte Einsatz von Biotests zu Überwachung der Grundwasserqualität gut geeignet ist. Weiterhin wird diskutiert, die Planung einer Sanierung nicht ausschließlich auf die Ergebnisse einer chemischen Analyse zu begründen. Vielmehr sollten alle anthropogen verursachten toxischen Verunreinigungen durch eine Kombination von chemischen und biologischen Tests identifiziert werden, um in einer folgenden Sanierung zielgerichtet berücksichtigt zu werden.

Abstract

This paper describes the use of a continuously online working biological test using luminescent bacteria for the control of groundwater remediation. The results of different other discontinuous biological tests using luminescent bacteria, algae, fish egg, daphnia and a bacterial genotoxicity test are illustrated and compared with the data of the on line biotest. The on line biotest appears to be very useful for monitoring the groundwater quality. Further on it is discussed, that the strategy for remediation should not only be based on results of chemical analysis alone. Rather the combination of chemical and biological tests should be used for the identification not only of the contaminants but of the toxic substances in the groundwater.

Einleitung

Erfolge von Maßnahmen zur Sanierung kontaminierter Grundwasservorkommen werden üblicherweise an der Fähigkeit zur räumlichen oder zeitlichen Begrenzung oder der Reduktion stofflicher Belastungen gemessen. Das hierfür betrachtete Substanzspektrum resultiert dabei vorrangig aus Erkundungen zu standortspezifischer Emissiongeschichte, regulativen Anforderungen, wie Geringfügigkeitsschwellen für ausgewählteStoffe und den verfügbaren methodischen Möglichkeiten zur Stoffanalytik. Zielsetzung von Grundwassersanierungsmaßnahmen ist im Regelfall eine über eine reine Schadenseingrenzung hinausgehende Sicherstellung zukünftiger Nutzungen des betroffenen Grundwasserkörpers. Die Betrachtung gängiger Nutzungen von Grundwasser wie Trinkwasser, Brauchwasser, Speisewasser für Seen oder Flüsse, Abwasser, Fischzuchtwasser oder Lebensraum für terrestrische oder aquatische Lebensgemeinschaften eröffnet die Option Tauglichkeitsanforderungen aus der Nutzung direkt für eine Erfolgskontrolle von Sanierungsmaßnahmen zu operationalisieren. Aus einer derartigen Perspektive rücken biologische Verfahren ins Blickfeld. Gereinigtes Grundwasser, das als Abwasser abgeführt wird, sollte beispielsweise mindestens kläranlagentauglich d.h. nicht bakterientoxisch sein; Grundwasser, das zur Befüllung zukünftiger Badeseen eingesetzt wird, sollte keine genotoxischen Wirkungen entfalten usw. Die Beurteilung möglicher schädlicher Effekte von belastetem Grundwasser kann sich nicht aus der alleinigen Betrachtung stoffanalytischer Bestimmungen ergeben. Dies zeigt schon ein Blick auf die regelmäßig anzutreffende Expositionssituation gegenüber Schadstoffmischungen oder die Sequenz von Ausgangskontamination und Abbauprodukten und die daraus resultierenden Kombinationswirkungen (ALTENBURGER *et al.* 1993, GRIMME *et al.* 1998).

In dieser Arbeit wird ein Teilvorhaben des BMBF geförderten Verbundprojektes SAFIRA – Sanierungsforschung in regional kontaminierten Aquifern vorgestellt (WEIß *et al.* 2001), das sich oben beschriebener Perspektive annimmt. Die Perspektiven einer Erfolgskontrolle von Sanierungsmaßnahmen für Grundwasserkontaminationen durch geeignete Kopplung von chemischen und biologischen Methoden werden exemplarisch für die SAFIRA-Pilotanlage in Bitterfeld entfaltet. Dabei wird neben der Nutzung biologischer Verfahren für Belastungscharakterisierungen und kontinuierliches Verfahrens-Monitoring, die Frage nach der Diagnose vorhandener Effektqualitäten und deren Einbindung in die Phase der Identifikation von Sanierungsbedarf erörtert.

Aufgrund hoher Umweltverschmutzung sowie punktueller und diffuser Schadstoffeinträge in das Grundwasser, werden häufig umfassende Sanierungsmaßnahmen nötig. Sind die jeweiligen Kontaminanten und mögliche Metaboliten bekannt, kann eine Sanierung spezifisch durchgeführt werden (ALTENBURGER *et al.* 1990, WALSH *et al.* 1999). In vielen Fällen handelt es sich bei verunreinigtem Grundwasser jedoch um unbekannte bzw. wenig bekannte Kontaminanten oder Gemische verschiedener Stoffe, die entfernt werden müssen (HAKSTEGE & VAN GELDERMALSEN 1998). Über mögliche Metaboliten ist in diesen Fällen ebenfalls wenig bekannt. Daher bestimmen die vor einer Sanierung durchgeführten chemischen Analysen häufig die Richtung bzw. Auswahl der sich anschließenden Sanierungsverfahren und -techniken. Ein Sanierungserfolg wird folglich über das Entfernen der chemisch nachgewiesenen Kontaminanten definiert (BIGG & JUDD 2002, SAWYER & LIEUALLEN-DULAM 1998). In einigen Fällen wird vor Einleiten oder auch während der Sanierung eine biologische Kontrolle in Form eines oder mehrerer Toxizitätstests (GUSTAVSON *et al.* 2000) oder einer Untersuchung von ökologisch wichtigen Organismen

durchgeführt (FORLIN & NORRGREN 1998). Diese, zum Teil zeitaufwendigen biologischen Tests, werden meist sporadisch durchgeführt. Der gleichzeitige Vergleich der Entgiftungseffizienz verschiedener Sanierungsverfahren mittels biologischer Tests ist zeit- und arbeitsaufwendig. Die vorliegende Arbeit stellt eine Methode vor, die die Kontrolle eines Sanierungserfolges, aber auch der Vergleich von neu entwickelten Sanierungsverfahren anhand automatisierter und kontinuierlicher ökotoxikologischer Effektanalyse durchführen kann. Diese Methode wurde im Rahmen eines Teilprojektes im Verbundvorhaben SAFIRA entwickelt (WEIß *et al.* 2001). Sowohl die ökotoxikologische Langzeitkontrolle der Grundwasserqualität vor, während und nach Sanierung, als auch die automatisierte biologische Analyse stellen Ziele dieses Teilprojektes dar.

Methoden

Die Überwachung neu entwickelter und zur Erprobung stehender Sanierungsverfahren in Bitterfeld erfolgt auf mehrere Arten. Neben der diskontinuierlichen chemischen Analyse werden zusätzlich biologische Tests zur Bestimmung der Toxizität des behandelten und unbehandelten Grundwassers durchgeführt. Die diskontinuierlichen biologischen Testverfahren beinhalten die Analyse von Wirkungen auf den bakteriellen Energiestoffwechsel und auf phyto-, neuro-, entwicklungs- und genotoxische Effektqualitäten. Zum Teil wurden die Tests aufgrund der spezifischen Anforderungen am Untersuchungsstandort entsprechend adaptiert. Dies war nötig, da die biologischen Tests für die Untersuchung von Oberflächenwasser bzw. Böden entwickelt wurden und nicht für Grundwasserproben mit einem hohen Anteil an leichtflüchtigen Halogenkohlenwasserstoffen (LHKW).

Es folgen kurze Prinzipbeschreibungen der eingesetzten biologischen Testverfahren.

Kurzzeit-Leuchtbakterientest: Test auf Störungen des bakteriellen Energiemetabolismus
Im -diskontinuierlichen- Leuchtbakterientest werden Bakterien der Art *Vibrio fisheri* mit einer Probe/ Umweltprobe über 15 bzw. 30 min inkubiert. Die Abnahme der Lumineszenz nach 15 bzw. 30 min Testdauer gegenüber einer unbehandelten Kontrolle gilt als Maß für die Beeinflussung des Energiemetabolismus der Bakterien durch unspezifisch oder spezifisch als Stoffwechselgifte wirkende Agentien. Der Test erfolgt in Anlehnung an DIN EN ISO 11348-1 (ANONYMOUS1999).
Neben diesem diskontinuierlichen Test wurde ein kontinuierlicher Leuchtbakterientest eingesetzt, der dem Prinzip des diskontinuierlichen Leuchtbakterientests folgt, allerdings für die Oberflächenwasserüberwachung konzipiert wurde (GERHARDT & PUTZGER 1999). Diverse Adaptationen wurden an diesem Gerät vorgenommen, um es für das kontinuierliche Monitoring von Grundwasser und Sanierungsverfahren in Bitterfeld nutzen zu können. Die technischen Veränderungen betreffen insbesondere:

- Möglichkeiten der abwechselnden und zeitnahen kontinuierlichen Analyse von verschiedenen Sanierungsverfahren bzw. Sanierungsreaktoren und des nativen Grundwassers;
- Erhöhung der Standzeiten des Gerätes;
- Steuerung und Fernüberwachung des Gerätes; sowie
- Datenbankentwicklung, GLP konforme Datenverarbeitung bzw. Datensicherung mit der Einbindung zusätzlicher wichtiger chemisch- physikalischen Parameter zur Probencharakterisierung (wie pH, Leitfähigkeit, H_2S, O_2 etc.) welche dem Leuchtbakterientest vorgeschaltet wurden.

Nicht alle am Standort in Bitterfeld vorhandenen Sanierungsreaktoren bzw. -verfahren können mit diesem automatisierten Biomonitor beobachtet werden. Einschränkungen, die die Beschränkung auf die Analyse von bestimmten Verfahren begründen, sind sowohl technisch, als auch verfahrensbedingt: zu geringe Durchflußgeschwindigkeit, Gefahr der Verockerung bzw. des Zusetzens der Zuleitungen, die periodische oder kontinuierliche Zudosierung von bakterientoxischen Substanzen in Sanierungsverfahren wie H_2O_2, Säuren o.ä..

Algenvermehrungstest: Test auf Phytotoxizität
Synchronisierte Kulturen der einzelligen Grünalge *Scenedesmus vacuolatus* wurden als Testorganismus genutzt. Endpunkt der toxischen Wirkung war die Bestimmung der Vermehrungshemmung während eines Generationenzyklus über 24 h (ALTENBURGER *et al.* 1990). Für das Testen von leichtflüchtigen Substanzen wurde der Test modifiziert. Diese Veränderungen betrafen die Nutzung von gasdichten Testgefäßen und von $NaHCO_3$ als Kohlenstoffquelle. Eine Veränderung der Reproduktionsleistung nach einer Generation im Vergleich zu einer unbehandelten Kontrolle kann integrativ Störungen diverser Prozesse anzeigen (GRIMME *et al.*, 1998) und wird als Hinweis für eine phytotoxische Wirkung der Probe gewertet.

Daphnientest: Test auf Neurotoxizität
Im Daphnientest wird der Effekt einer Probe/ Umweltprobe auf die Mobilität von Daphnien-Neonaten (*Daphnia magna*) untersucht. Die Neonaten werden über einen Zeitraum von 48 h mit der Probe inkubiert und der Effekt in Form von Bewegungsunfähigkeit bzw. -störungen und Tod nach 24 h und 48 h beobachtet und mit Kontrollansätzen verglichen. Die Beeinflussung der Probe auf die Bewegungsfähigkeit gilt u.a. als Indikator für mögliche neurotoxisch wirkende Agentien. Die Versuchsdurchführung erfolgt in Anlehnung an DIN EN ISO 6341 (ANONYMOUS1996a).

Fischeientwicklungstest mit dem Zebrabärbling: Test auf Entwicklungstoxizität
Im Fischeitest wird der Effekt einer Probe/ Umweltprobe auf die allgemeine Entwicklung vom Fischei des Zebrafisches (*Danio rerio*) bis zum Schlupf des Jungfisches untersucht. Dabei werden die Fischeier direkt mit der Probe über einen Zeitraum von 48 h inkubiert und die Entwicklung anhand spezifischer Endpunkte wie Herzschlag, Somitenbildung, Schwanzentwicklung, Schlupfrate etc. beobachtet und dokumentiert. Getestet wird damit die allgemeine und spezifische Entwicklungstoxizität von Testgütern. Die Durchführung erfolgt in Anlehnung an DIN 38415-6 (ANONYMOUS2001).

umu-Test: Test auf Genotoxizitätstest
Im umu- Kurzzeitgenotoxizitätstest wird der gentechnisch veränderte Bakterienstamm *Salmonella typhimurium* TA1535/ pSK1002 (Entero-bacteriaceae) gegenüber einer Probe für einen Zeitraum von 4 Stunden exponiert. Bei einer genotoxischen Wirkung der Probe kommt es sowohl zur Aktivierung von zellulären Reparaturmechanismen (SOS-System) als auch zur Induktion eines Reportergens, das für ein leicht zu quantifizierendes Enzym codiert. Die Quantität des gebildeten Enzyms, welche kolorimetrisch bestimmt wird, gilt als Testkriterium für den Nachweis einer genotoxischen Wirkung. Die differenziert zu bewertende Zytotoxizität der Probe wird gleichzeitig durch die Wachstumsrate der Bakterien bestimmt. Der Test wurde in Anlehnung an DIN 38415-3 durchgeführt (ANONYMOUS1996b).

Die untersuchten Grundwasserproben werden für die unterschiedlichen Testsysteme im Verhältnis 1:2 verdünnt. Solange nicht explizit anderes angegeben wird, beziehen sich

daher sämtliche Angaben auf 50 % verdünnte Proben.

Ergebnisse

Charakterisierung der unspezifischen Toxizität des Grundwassers am Versuchsstandort in Bitterfeld

Vor dem Einsatz einer regelmäßigen toxischen Analyse und einer Beurteilung über die Verringerung der Toxizität durch den Einsatz spezieller Sanierungsverfahren, war eine Charakterisierung des die Forschungsanlage anströmenden kontaminierten Grundwassers nötig. Mit Hilfe des diskontinuierlichen Leuchtbakterientests wurden die toxischen Effekte des Grundwassers auf Leuchtbakterien am Sanierungsstandort in Bitterfeld vor (d.h. im Anstrom) und auf dem Gelände der Anlage untersucht. Auf Abb. 1a & 1b sind die für die entsprechenden Pegel gemessenen Leuchtbakterientoxizitäten entsprechend den Intensitäten in unterschiedlich großen Kreisen dargestellt. Erkennbar ist, daß hohe Toxizitäten an allen Pegeln vor bzw. nördlich der SAFIRA Anlage gemessen wurden. Erst auf dem Gelände und östlich der SAFIRA Anlage verringert sich die Bakterientoxizität. Abb.1b zeigt die Toxizitäten und die korrespondierenden Messtellen auf dem Gelände der Anlage im Detail. Die Daten aus den chemisch- analytischen Untersuchungen im Verbundprojekt ergaben Verteilungen an leichtflüchtigen, chlorierten Kohlenwasserstoffverbindungen (LHKW) (Weiß *et al.* 1997, Weiß *et al.* 2001), die nicht im Widerspruch zur Annahme von unspezifisch wirkenden Stoffen als Ursache der beobachteten Toxizitäten stehen. Die Sanierungsverfahren, die zur Erprobung in der Pilotanlage eingesetzt werden, konzentrieren sich weitgehend auf die Reduktion des massenbilanzmäßig dominierenden Monochlorbenzols.

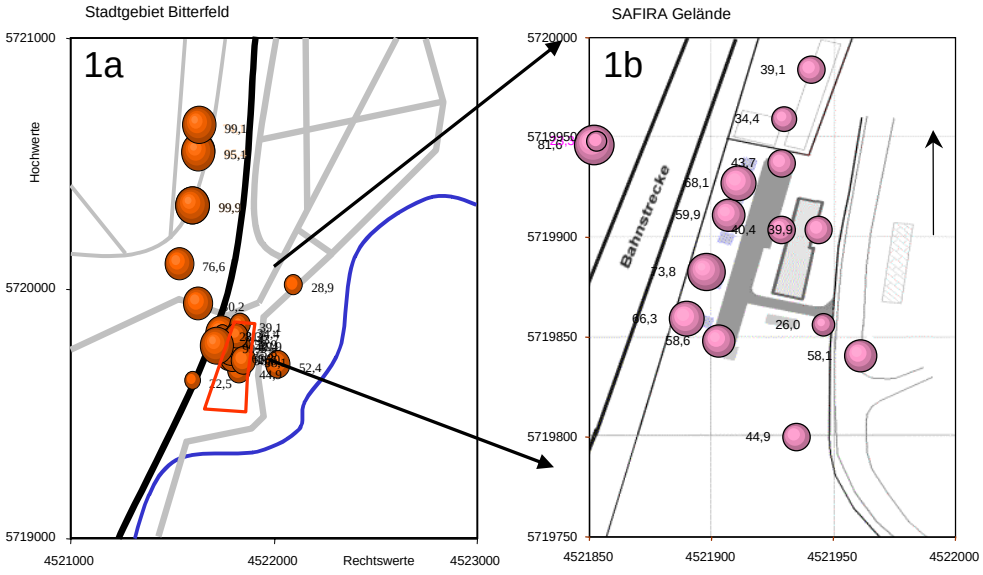


Abb. 1: Leuchtbakterientoxizitäten an ausgewählten Pegeln im SAFIRA Grundwasseranstrom (1a) und auf dem SAFIRA Gelände (1b); die Kreisgröße gibt die prozentuale Lumineszenzhemmung an

Kontinuierliches Monitoring des anströmenden Grundwassers an der Pilotanlage

Die Pilotanlage besteht u.a. aus fünf Brunnen, in denen verschiedene Sanierungsverfahren erprobt werden. Grundwasser (in Folge auch “Horizontalbrunnen” genannt), welches diese Brunnen anströmt und in Horizontalbrunnen aufgefangen wird, wird durch Sanierungsreaktoren gepumpt, um dann weiter analysiert zu werden.

Zunächst wurden die Toxizitäten des Grundwassers der einzelnen Horizontalbrunnen charakterisiert, welche durch die verschiedenen zur Erprobung stehenden Sanierungsverfahren behandelt werden sollten. Diese Analysen wurden sowohl mit diskontinuierlichem als auch mit automatisiertem kontinuierlichen Leuchtbakterientest durchgeführt. In beiden Tests zeigte sich eine hohe Leuchtbakterientoxizität in den 1:2 verdünnten Grundwasserproben. Dies steht in guter Übereinstimmung mit den Ergebnissen der vorausgegangenen Standortcharakterisierung und den chemischen Analysen.

Die Ergebnisse des automatisierten und kontinuierlichen Leuchtbakterien- Biotests zeigt eine deutlich andere Datenqualität, im Vergleich zum diskontinuierlichen Leuchtbakterientest (Abb.2/ 3). Obwohl die meßbaren Effekte der einzelnen Horizontalbrunnen wie erwartet in der gleichen Größenordnung lagen, zeigte sich, daß die Grundwässer der einzelnen Horizontalbrunnen (in beiden angewandten Tests) immer noch leicht voneinander zu unterscheiden waren. Eine Beobachtung, die auch mit den chemisch analytischen Ergebnissen vor Ort gemacht wurde. Beim automatisiertem kontinuierlichen Leuchtbakterientest war dieser Unterschied allerdings deutlicher zu erkennen.

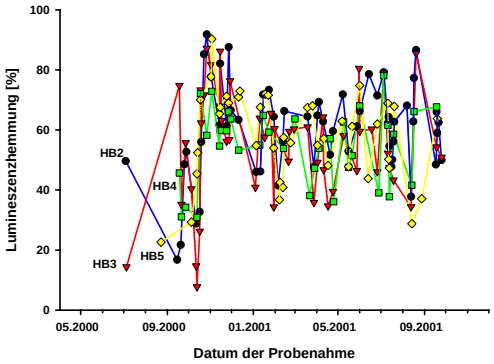


Abb.2: Leuchtbakterientoxizitäten von vier Horizontalbrunnen mittels diskontinuierlichem Test im Zweiwochenrhytmus gemessen

Die Abb.3 zeigt repräsentative kontinuierliche Datenaufnahmen für vier der fünf Horizontalbrunnen über einen Zeitraum von 80 h. Die inhibierenden Effekte des Grundwasser des einen Horizontalbrunnen liegt bei ca. 80 %, die toxischen Effekte der anderen Brunnen zum Teil deutlich bzw. gut unterscheidbar darunter.

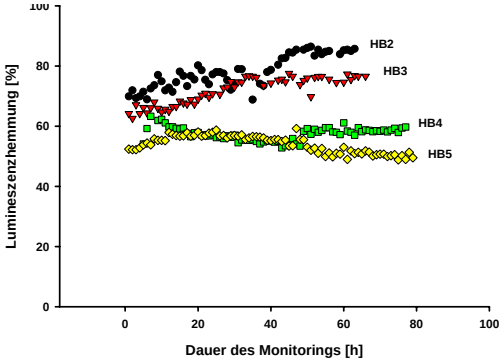


Abb.3: Leuchtbakterientoxizitäten von vier Horizontalbrunnen mittels automatisiertem/kontinuierlichem Biotest gemes-sen

Da ein Gerät für die gleichzeitige Analyse von fünf Horizontalbrunnen und zwölf verschiedenen Sanierungsreaktoren genutzt wird, wurden die vier Horizontalbrunnen und einige ausgewählte Reaktoren über mehrere Monate im Wechsel beobachtet.

Eine Zusammenfassung sämtlicher bisher erfaßten Daten -in Form einer Regressionsgeraden- der toxischen Effekte des Grundwassers auf Leuchtbakterien von einem Jahr zeigt, daß für alle vier Horizontalbrunnen kaum eine Änderung der Effekte auf Leuchtbakterien erkennbar ist (Abb. 4). Einzig der Horizontalbrunnen 2 (HB2) zeigte eine geringfügige Erhöhung von ca. 10 % im Laufe des Beobachtungszeitraums.

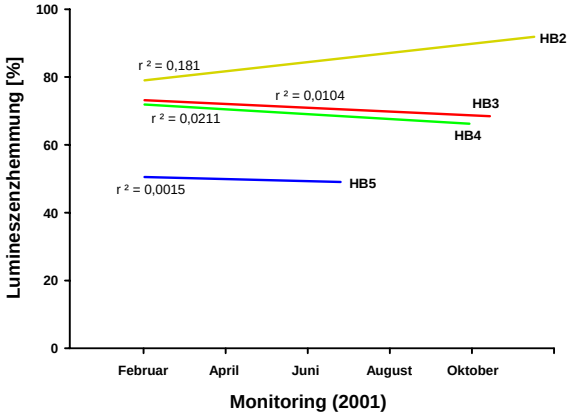


Abb. 4: Entwickungsvergleich der Leuchtbakterientoxizitäten über einen Zeitraum von bis zu 10 Monaten der beobachteten anströmenden Grundwasser an der SAFIRA Anlage (in diesen Zeiträumen wurden stichprobenartige Analysen mit dem kontinuierlichen Leuchtbakterientest durchgeführt), dargestellt als Regressionsgeraden.

Kontinuierliche Überwachung eines mit Aktivkohle befüllten Reaktors

Für die Überprüfung der Tauglichkeit des angewandten automatisierten kontinuierlichen Leuchtbakterientests zur Beurteilung einer erfolgreichen Dekontamination des Grundwassers wurde ein auf Aktivkohle basierendes Sanierungsverfahren beobachtet, welches sämtliche bislang chemisch- analytisch nachgewiesenen und quantitativ am höchsten vorkommenden organischen Schadstoffe entfernen sollte. Nach der chemischen Überprüfung des Grundwassers, welches den Testreaktor durchflossen hatte, wurden keine LHKW mehr nachgewiesen. Daher sollte auch im Leuchtbakterientest keine Toxizität nachweisbar sein. Diese Überprüfung diente somit als eine interne Kontrolle. Abb. 5 zeigt eine Darstellung der Messergebnisse von mehrtägigen Meßkampagnen im Laufe eines Jahres als Differenzmessung von Horizontalbrunnen- und Reaktorwasser, also unbehandeltem und behandeltem Grundwasser. Die Ergebnisse der Toxizitätsbestimmungen zeigten, daß es zu einer absoluten Entfernung von Stoffen, die den Energiemetabolismus der Bakterien stören, durch dieses Verfahren kam. Die Effektivität der Entgiftung durch dieses Verfahren nahm auch in einem folgendem mehrmonatigen Beobachtungszeitraum nicht ab. Da im Leuchtbakterientest ein Effekt erst ab einer Hemmung der Lumineszenz von 20 % und höher als toxisch bewertet wird, ist die Erhöhung von 0 auf ca. 15 % im Januar 2002 (Abb.5) noch nicht relevant.

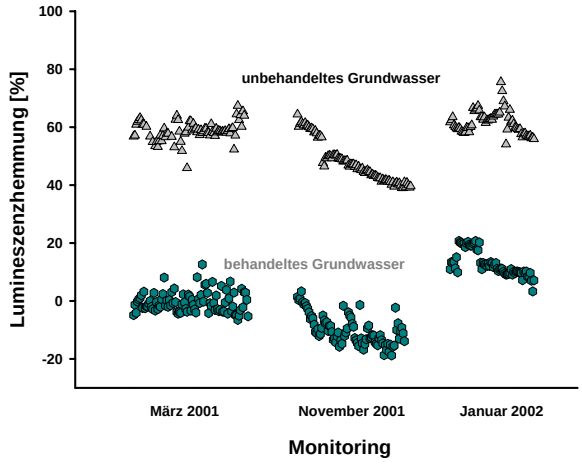


Abb. 5: Toxizitäten von Grundwasser, welches mittels eines auf Aktivkohle basierendem Verfahren gereinigt wird. Stichprobenartige Messungen von 4-5 Tagen im Zeitraum eines Jahres.

Detoxifizierungseffizienz anderer Verfahren/Reaktoren

Neben der Überwachung der Toxizitäten der Horizontalbrunnen und des durch Aktivkohle sanierten Grundwassers wurde auch die Effektivität der anderer Sanierungsverfahren bzw. einzelner Reaktoren über einen längeren Zeitraum beobachtet. Es zeigte sich eine unterschiedliche Stabilität bzw. Effizienz der Verfahren, die toxischen Effekte auf die Leuchtbakterien zu entfernen oder zu verringern.

Abb. 6 zeigt die Ergebnisse der Beobachtung eines zweiten dem oben beschriebenen allerdings unähnlichem Aktivkohleverfahrens. In Abb. 7 ist ein Verfahren dargestellt, welches auf der katalysierten Dechlorierung der chlorierten Kohlenwasserstoffe und dem folgenden mikrobiellen Abbau der Reaktionsprodukte in einem Aquiferreaktor beruht. Bei diesem Verfahren wurde die Toxizität des Grundwassers untersucht, welches aus dem Aquiferreaktor floß. Nach einer anfangs vollständigen Entgiftung steigt bei beiden Untersuchungen die gemessene Toxizität im Laufe des Zeitraumes von April- Dezember 2001 bzw. Februar 2002 an.

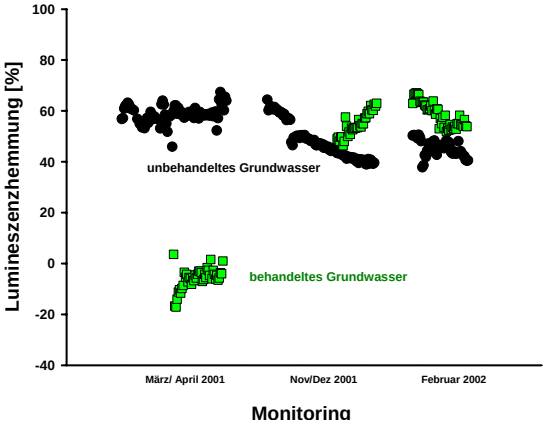


Abb. 6: Toxizitäten von Grundwasser, welches mittels eines zweiten -von Abb.5 verschiedenen Aktivkohle-Verfahrens gereinigt wird. Stichprobenartige Messungen von 4-5 Tagen im Zeitraum eines Jahres.

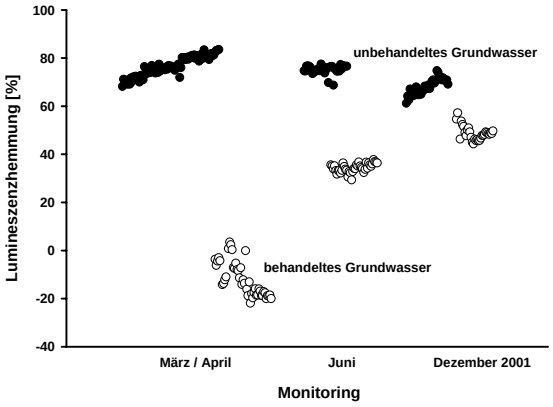


Abb. 7: Verlauf der Toxizitäten des Grundwassers eines auf der Katalyse der Kontaminanten basierendes Verfahren im Zeitraum eines Jahres im Vergleich zum unbehandelten Grundwasser (stichprobenartige Messungen von 4-5 Tagen).

Die parallel durchgeführte regelmäßige Kontrolle der Toxizitäten der besprochenen Verfahren mit Hilfe des diskontinuierlichen Leuchtbakterientests zeigte eine ähnliche Entwicklung der Effektivität der Sanierungsverfahren bzw. der Horizontalbrunnen aber mit der oben auch schon besprochenen schlechteren Auflösung und höheren Streuung (Daten nicht dargestellt).

Treten neben der Bakterientoxizität noch weitere toxische Effektqualitäten auf?

Wie in Abb. 6 dargestellt, verringerte sich die Effizienz des einen Aktivkohleverfahrens bzw. des nachgeschalteten Aquiferreaktor im Laufe der Beobachtungszeit zusehends. Dies stand nun im Gegensatz zu den Einschätzungen, die durch die chemischen Analysen gestützt wurden. Diese hatten bisher kein “Durchbrechen” der Schadstofffront im Aktivkohlereaktor feststellen können. Als Folge stellte sich die Frage nach der Ursache der gemessenen Leuchtbakterientoxizität. Deswegen wurden verschiedene andere biologische

Tests mit diversen Testorganismen angewandt, um eventuell Rückschlüsse auf andere bisher nicht berücksichtigte Effektqualitäten und damit andere noch nicht bekannte Stoffgruppen darstellen zu können. Die Ergebnisse dieser Analysen sind in Abb. 8a/b dargestellt.

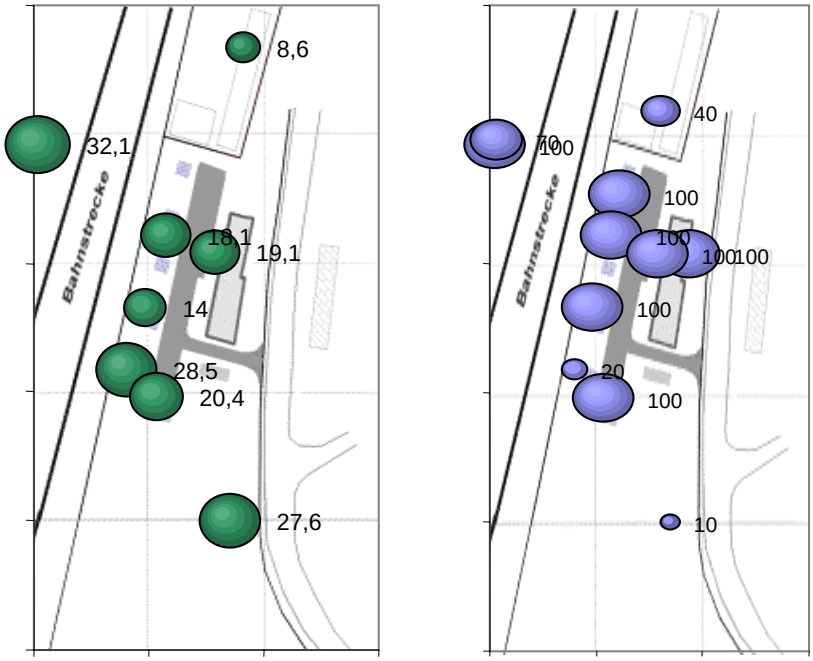


Abb. 8: Toxische Effekte des Grundwassers auf dem Gebiet der SAFIRA-Anlage auf Algen (8a) und Daphnien (8b); Angaben in % Vermehrungshemmung bzw. Immobilisierung.

Sie zeigen - es wurden für diese Untersuchungen zunächst nicht die Sanierungsreaktoren selbst, sondern alle Pegel auf dem SAFIRA Gelände untersucht -,daß die Ergebnisse zwischen den biologischen Tests sehr unterschiedlich waren.Der Algentest beispielsweise indiziert eine verhältnismäßig geringe Phytotoxizität von 10- 30 % des 1:2 verdünnten Grundwassers der verschiedenen Pegel (Bild 8a). Dagegen führten die Daphnientests zu einer 10-100 % Immobilisierung, wobei die Anzahl der Pegel mit 100 % Immobilisierungsrate stark überwog (Bild 8b). Sehr ähnlich war das Ergebnis der Fischeientwicklungstests. Hier wurde allerdings bei allen Messtellen eine 100 % Toxizität gemessen, weswegen hier auf eine graphische Darstellung verzichtet wurde. Die Tests zur Bestimmung einer möglichen genotoxischen Wirkung (umu-Test) zeigte für das Grundwasser der jeweiligen Messpegel keine Effekte an.

Aufgrund der stark differenzierten Toxizitätsbefunde stellt sich die Frage, ob die chemisch-analytisch nachgewiesenen bekannten und quantitativ bedeutensten Schadstoffe, darunter besonders das Monochlorbenzol, tatsächlich allein für diese differenzierten Effektqualitäten in den verschiedenen biologische Testverfahren verantwortlich sein können. Eine Analyse der Konzentrations-Wirkungs-Beziehungen für Monochlorbenzol (MCB) in den einzelnen biologischen Testverfahren und die anschließende Nutzung zur Modellierung der am jeweiligen Standort erwartbaren Effekte durch die analytisch bestimmten Konzentrationen bestärkt diese Zweifel an Monochlorbenzol als hauptsächlich wirksamer Komponente noch.

Diskussion

Im Folgendem werden zunächst die Vor- und Nachteile eines automatisierten und kontinuierlichen biologischen Monitorings gegenüber einer diskontinuierlichen Biotestung erörtert und eine Diskussion der Tauglichkeit von automatisierten Verfahren zur Überwachung von Energiemetabolismusgiften in der Kontrolle von Sanierungsverfahren geführt. Der Erörterung der Validität und Reflektion der erfassten Effekte im Hinblick auf das Vorliegen weiterer Effektqualitäten folgt eine Diskussion über die Einbeziehung toxikologischer Überlegungen bei der Plannung von Sanierungsvorhaben. Dies schließt sowohl die Identifikation von Sanierungsbedarf, als auch die folgende technische Sanierungsplanung und -ausführung ein.

Wie mit den Ergebnissen in Abb. 3 und 4 dargestellt, ist der kontinuierlich und automatisiert arbeitende Bakterienstoffwechselaktivitätstest mit *Vibrio fischeri* (in Folge nur automatisierter Leuchtbakterientest genannt) in Bezug auf die Nutzung für Sanierungskontrolle dem diskontinuierlich arbeitenden Verfahren überlegen. Dies hat mehrere Gründe. Die Daten des aus den diskontinuierlichen Leuchtbakterientests zeigen zwar quantitativ vergleichbare Messergebnisse, sind aber schlechter interpretierbar, da sie erheblich größere Streuungen aufweisen. Für diese Streuungen könnten Probleme in der Reproduzierbarkeit durch Pipettierungenauigkeiten, messtechnische Schwankungen etc. vermutet werden. Allerdings zeigen Verfahrenskontrollen mit Positivkontrollen keine Plausibilität für eine derartige Annahme. Wesentlich wahrscheinlicher ist, daß Einflüsse wie die Probenahme, die Probenlagerung oder die Probenbehandlung und ähnliches die Reproduzierbarkeit der Analysen für die vorliegende Probensituation erschweren. Bei einem automatisiertem Biotest bestehen diese Art der Probleme nicht. Die Überwachung von Kläranlagen (MITZ & GIESY 1985, ARULDOSS & VIRARAGHAVAN 1998), Deponie/ Minensickerwässern (KARUPPIAH & GUPTA 1997), (LEBLOND & DUFFY 2001), Fabrikeinleitungen (MIDDAUGH *et al.* 1997), Flußsedimenten (GUZZELLA 1998) Sediment-Porenwasser (KARUPPIAH & GUPTA 1996) ist seit längerer Zeit neben der chemischen Kontrollanalytik eingeführt. Bedingt durch die Messung mit einem diskontinuierlichen biologischem bzw.chemischen Test können Störungen in den Sanierungsverfahren (Pumpen- bzw. Reglerausfall, Fehldosierungen von Hilfssubstanzen, Verockerungen oder verstopfte Leitungen) oder auch die Abnahme der Sanierungseffizienz nicht in gleicher Präzision bzw. in vergleichbarer Zeitnähe zur Auslösung einer Änderung detektiert und damit überwacht werden, wie in einem automatisiertem und kontinuierlichem Test. Andere Nachteile beim Gebrauch und Durchführung von nicht automatisierten Biotests sind in den oft hohen personellen und zeitlichen Arbeitsaufwendungen zu sehen. Zur Lösung der genannten Probleme werden existierende biologische Testverfahren häufig mit dem Ziel einer Erhöhung des Probendurchsatzes, größerer Messgenauigkeit etc. miniaturisiert und vereinfacht (SCHMITZ *et al.* 1998, FIEHN *et al.* 1997) oder durch den Einsatz von Biosensoren ersetzt, wenn es sich nur um einige wenige Substanzen handelt, die überwacht werden müssen (FENNOUH *et al.* 1997).

Eine automatisierte Analyse der Toxizität besitzt diese Nachteile dagegen nicht. Die hier dargestellten Ergebnisse (Abb. 3, 5, 6, 7) zeigen, daß der an die Verhältnisse der Grundwasseranalyse adaptierte und umgebaute automatisierte Leuchtbakterientest technisch gesehen gut für eine Überwachung geeignet ist. Dies gilt sowohl für die Überwachung der Grundwasserkontamination, als auch der Effizienz und Effektivität einzelner Sanierungsreaktoren bzw. -verfahren. In den meisten Sanierungsfällen, bei denen neue Verfahren getestet werden, kann der automatisierte Leuchtbakterientest damit oft eine verbesserte Alternative gegenüber einem diskontinuierlichen Leuchtbakterientest

darstellen.

Die Frage bei jedem eingesetzten Biotestverfahren -wie auch bei jeder anderen chemischen Stoffanalytik- ist allerdings die Bedeutung der Befunde. Jeder biologische Test kann durch milieu- oder verfahrensbedingte Störungen zu Fehleinschätzungen führen. Hohe Gehalte bestimmter Salze, Ionen, Temperatur, Leitfähigkeit, pH, H_2S, O_2 etc. (COOK *et al.* 2000, HINWOOD & MCCORMICK 1987, BULICH *et al.* 1981, KREBS 1992, WHITEMAN *et al.* 1996, KORNER *et al.* 2001, KISHINO & KOBAYASHI 1995, BROUWER & MURPHY 1995) können die Differenzierung und Identifizierung von anthropogen verursachten toxischen Agentien von den natürlich vorkommenden toxischen Milieufaktoren erschweren. Deswegen muß zum Einen sichergestellt werden, daß nicht falsch positive Toxizitäten befundet werden und zum Anderen, daß die angewandten biologischen Testsysteme für die untersuchten Verfahren überhaupt relevant bzw. genügend sensitiv sind (SCHMITZ *et al.* 1999, BACKHAUS *et al.* 1997). Stoffe, die für Leuchtbakterien toxisch sind, müssen nicht unbedingt auch für Daphnien oder Fische toxisch sein (LEBLOND & DUFFY 2001, PETER *et al.* 1995). Ein biologisches Testverfahren, welches eine einzige Effektqualtität wie den Energiemetabolismus abbildet, kann durch die Erfassung weiterer Effektqualitäten etwa Neuro-, Phyto-, Gen oder entwicklungstoxischer Effekt im Hinblick auf standortspezifische Relevanz validiert werden.

Ein zusätzliches Problem stellt sich bei der Beurteilung von Toxizitäten, welche nicht durch die chemisch-analytisch bestimmte(n) Hauptkontaminante(n) erklärt werden kann, wie das in Abb. 6 gezeigt wurde. Bisher nicht identifizierte bzw. chemisch nicht analysierte Stoffe wie z.B. Metaboliten, welche erst im Sanierungsvorgang selbst entstehen bzw. synthetisiert werden, können für toxische Effekte verantwortlich sein. Ebenfalls können Substanzen oder Substanzgemische in Konzentrationen vorliegen, die evt. in Kombination miteinander toxisch auf den Testorganismus wirken (ALTENBURGER *et al.* 2000, FAUST *et al*. 2000, BACKHAUS *et al.* 2000, GRIMME *et al.* 2000).

Eine aufgrund von Massenbilanzen definierte Hauptkontaminante muß also nicht prinzipiell auch die toxischste Substanz sein. Als Beispiel kann das Grundwasser, welches im Horizontalbrunnen 5 auf dem SAFIRA Gelände analysiert wird, angeführt werden. Dieses weist die höchsten Konzentrationen der Hauptkontaminante Monochlorbenzol am Standort auf. Die hier gemessene Leuchtbakterientoxizität ist entgegen der Erwartung die geringste von allen untersuchten Grundwässern aus den Horizontalbrunnen.

Ebenfalls sind falsch negative Befunde möglich. Falls es bei einem Sanierungsverfahren nicht zu einer vollständigen Entfernung oder nur zu einer teilweisen Verringerung der Toxizität kommt, sollte diese Toxizität im Idealfall immer auf die auslösenden Substanzen zurückgeführt werden, d.h. die toxischen Substanzen identifiziert werden können. Im Prinzip kann durch geeignete Probenmanipulation wie bespielsweise die Austreibung flüchtiger Verbindungen und daran gekoppelte biologische Tests eine Identifizierung der toxischen Substanzen in einem Gemisch welches eine Umweltprobe immer darstellt, stattfinden. Für diese Analysen bestehen bereits auf anderen Gebieten ausgearbeitete Verfahrenvorschläge und Methoden, welche auch in einigen Studien bereits erfolgreich durchgeführt wurden (BRACK *et al.* 1999, GUSTAVSON *et al.* 2000, DEANOVIC et al. 1999, MICHAELIDOU et al. 1995, BAILEY *et al.* 1999).

Für den Planungsvorgang zukünftiger Sanierungsvorhaben könnte eine Identifizierung nicht allein der bekannten Kontaminanten, sondern auch aller toxische bedeutenden Substanzen bereits in der ersten Phase der Analyse des Sanierungsbedarfes eine

zielorientierte Adaptation bzw. Neuentwicklung und Verbesserung von Sanierungsverfahren ermöglichen. Dieser Verfahrensvorschlag steht allerdings im Widerspruch zur derzeit üblichen Verfahrensweise. Aufgrund der historischen Entwicklung und der hohen Qualität der chemischen Wasseranalytik wurden und werden Erfolge von Sanierungsvorhaben bzw. Sanierungsverfahren hauptsächlich danach beurteilt, ob sie die Hauptkontaminanten oder konsentierten Problemstoffe, welche chemisch nachgewiesen wurden, verringern oder ganz entfernen können. In dem Fall, in dem bei akuten Schadensfällen oder punktuellen Verunreinigungen die Kontaminanten bekannt sind und es sich nur um eine oder einige wenige bekannte Substanzen handelt, ist dieses Vorgehen möglicherweise ausreichend. Problematisch kann es insbesondere bei Grundwasserkontaminationen sein, deren Ursprung und Verursacher nicht bekannt sind oder deren Verunreinigung sich aus vielen verschiedenen Substanzen zusammen setzt. Wird dieses Grundwasser chemisch analysiert, wird üblicherweise nach den vermuteten oder auch dominierenden Problemstoffen gesucht. Die vorhandenen human- und umwelttoxikologischen Daten werden gesichtet und die Sanierungsverfahren daraufhin entsprechend entwickelt. Ein Sanierungsziel wird damit als das Entfernen der bekannten und als problematisch definierten Stoffe betrachtet.

Nach dem politisch- regulativem Verständnis wie es sich etwa in den LAWA Geringfügigkeitsschwellen ausdrückt, ist Grundwasser ein Gut, das für alle möglichen zukünftigen Nutzungen zu schützen ist. Eine Folge dieses Grundsatzes ist, daß die Überwachung und evt. nötige Sanierung des Grundwassers im Grunde auch nutzungsabhängig betrachtet werden muß. Für die Analyse von Wirkqualitäten wie Geno-, Neuro-, Bakterien-, Entwicklungs- und Phytotoxizität bestehen bereits anerkannte Verfahren, die für die Beurteilung der zukünftigen Nutzung als Trinkwasser, Badewasser, Abwasser, Lebensraum für Organismen etc. genutzt werden können.

In den Fällen mit einer komplexen Kontamination des Grundwasser gilt damit: Wenn man die Frage nach der Reduktion der Toxizität durch eine etwaige Sanierung beantworten will, reicht die physikochemische Analytik allein nicht mehr aus. Vielmehr werden spezifische bzw. relevante biologische Testverfahren benötigt, die abhängig von der geplanten Nutzung des kontaminierten Grundwassers ausgewählt werden müssen, um den Sanierungserfolg im Hinblick auf eine erfolgreiche Toxizitätsreduktion überwachen zu können.

Zusammenfassung

Zusammenfassend läßt sich feststellen, daß der vorrangig untersuchte automatisierte Leuchtbakterientest nach den vorliegenden Erfahrungen gut für eine biologische Grundwassersanierungskontrolle geeignet ist. Ebenfalls eignet er sich für eine Langzeit-Verfahrenskontrolle bzw. -überwachung auch anderer Sanierungsverfahren. Nötig ist allerdings immer eine interne Kontrolle auf milieubedingte Störgrößen und auf Relevanz der Messergebnisse in Bezug auf die geplante zukünftige Nutzung des sanierten Gutes.
Der Einsatz zur Bewertung eines Sanierungserfolges von kontaminiertem Grundwasser wird empfohlen, wenn die Art der Kontamination unspezifische Toxizitäten oder Energiestoffwechselgifte vermuten läßt oder das Nutzungsziel mit Leuchtbakterientesten ausreichend gesichert werden kann.
Die Identifizierung der toxischen Substanzen vor Beginn einer Sanierung könnte die Entwicklung zielorientierter Sanierungsverfahren im Hinblick auf unerwünschte Gifteffekte oder zukünftige Nutzungen verbessern helfen.

Danksagung

Wir danken dem Bundesministerium für Bildung und Forschung (bmb+f) für die Finanzierung dieses Teilprojektes (D 2.1) des SAFIRA Verbundprojektes - Förderkennzeichen: 02WT9949/3

Literatur

ALTENBURGER, R., BOEDEKER, W., FAUST, M., AND GRIMME, L.H.(1990). Evaluation of the isobologram method for the assessment of mixtures of chemicals. Combination effect studies with pesticides in algal biotests. *Ecotoxicology And Environmental Safety* **20**: 98-114.

ALTENBURGER,R., BOEDEKER, W., FAUST, M., AND GRIMME, L.H. (1993) Aquatic Toxicology, Analysis of Combination Effects. *Handbook of Hazardous Materials* (Academic Press): 15- 27.

ALTENBURGER, R., BACKHAUS, T., BOEDEKER, W., FAUST, M., SCHOLZE, M., GRIMME, L.H. 2000. Predictability of the toxicity of multiple chemical mixtures to *Vibrio fischeri*: Mixtures composed of similarly acting chemicals. *Environ Toxicol Chem,* **19**: 2341-2347.

Anonymous (1996), DIN 38415-3, Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung - Suborganismische Testverfahren (Gruppe T) - Teil 3: Bestimmung des erbgutverändernden Potentials von Wasser mit dem umu-Test (T 3) 12: 1996.

Anonymous (1996), DIN EN ISO 6341, Wasserbeschaffenheit - Bestimmung der Hemmung der Beweglichkeit von Daphnia magna Straus (Cladocera, Crustacea) - Akuter Toxizitäts-Test (ISO 6341:1996); Deutsche Fassung EN ISO 6341 6: 1996.

Anonymous (1998), (DIN EN ISO 11348-1, Wasserbeschaffenheit - Bestimmung der Hemmwirkung von Wasserproben auf die Lichtemission von Vibrio fischeri (Leuchtbakterientest) - Teil 1: Verfahren mit frisch gezüchteten Bakterien (ISO 11348-1:1998); Deutsche Fassung EN ISO 11348-1: 1998.

Anonymous (2001), DIN 38415-6, Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung - Suborganismische Testverfahren (Gruppe T) - Teil 6: Giftigkeit gegenüber Fischen; Bestimmung der nicht akut giftigen Wirkung von Abwasser auf die Entwicklung von Fischeiern über Verdünnungsstufen (T 6) 9: (2001).

ARULDOSS,J.A.; VIRARAGHAVAN,T. (1998). Toxicity testing of refinery wastewater using Microtox. *Bulletin Of Environmental Contamination And Toxicology*, **60** (3): 456- 463.

BACKHAUS,T.; FROEHNER,K.; ALTENBURGER,R.; GRIMME,L.H. (1997). Toxicity testing with Vibrio fischeri: A comparison between the long term (24 h) and the short term (30 min) bioassay. *Chemosphere*, **35** (12): 2925- 2938.

BACKHAUS, T., ALTENBURGER, R., BOEDEKER, W., FAUST, M., SCHOLZE, M., GRIMME, LH. 2000. Predictability of the toxicity of a multiple mixture of dissimilarily acting chemicals *to Vibrio fischeri*. *Environ Toxicol Chem,* **19**: 2348-2356.

BAILEY, H. C., ELPHICK, J. R., POTTER, A., CHAO, E., KONASEWICH, D., AND ZAK, J. B. (1999). Causes of toxicity in stormwater runoff from sawmills. Environ Toxicol Chem 18: 1485-1491.

BIGG, T. AND JUDD, S.J. (2002). Electrochemical monitoring of water remediation by metallic iron. *Journal Of Applied Electrochemistry* **31**: 1339-1344.

BRACK, W., ALTENBURGER, R., ENSENBACH, U., MODER, M., SEGNER, H., AND SCHÜÜRMANN, G. (1999). Bioassay-directed identification of organic toxicants in river sediment in the industrial region of Bitterfeld (Germany) - A contribution to hazard assessment. Archives Of Environmental Contamination And Toxicology 37: 164-174.

BROUWER,H AND MURPHY,T (1995). Volatile sulfides and their toxicity in freshwater sediments. *Environ Toxicol Chem* **14** (2): 203- 208.

BULICH,A.A.; GREENE,M.W.; ISENBERG,D.L. (1981). Reliability of the bacterial luminescence assay for determination of the toxicity of pure compounds and complex effluents. Aquatic Toxicology and Hazard Assessment- Fourth Conference: 338- 347.

COOK,S.V.; CHU,A.; GOODMAN,R.H. (2000). Influence of salinity on Vibrio fischeri and lux-modified Pseudomonas fluorescens toxicity bioassays. *Environ Toxicol Chem* **19** (10): 2474- 2477.

DEANOVIC, L., CONNOR, V. M., KNIGHT, A. W., AND MAIER, K. J. (1999). The use of bioassays and toxicity identification evaluation (TIE) procedures to assess recovery and effectiveness of remedial activities in a mine drainage-impacted stream system. *Archives Of Environmental Contamination And Toxicology* **36**: 21-27.

FAUST, M., ALTENBURGER, R., BACKHAUS, T., BOEDEKER, W., SCHOLZE, M., GRIMME, L.H. 2000. Predictive Assessment of the Aquatic Toxicity of Multiple Chemical Mixtures. *J. Eur. Qual*, **29**: 1063-1068.

FENNOUH,S.; CASIMIRI,V; BURSTEIN,C. (1997). Increased paraoxon detection with solvents using acetylcholinesterase inactivation measured with a choline oxidase biosensor. *Biosensors & Bioelectronics* **12** (2): 97- 104.

FIEHN,O.; VIGELAHN,L.; KALNOWSKI,G.; REEMTSMA,T.; JEKEL,M. (1997). Toxicity-directed fractionation of tannery wastewater using solid-phase extraction and luminescence inhibition in microtiter plates. *Acta Hydrochim Hydrobiol* **25** (1): 11- 16.

FORLIN, L. AND NORRGREN, L.(1998). Physiological and morphological studies of feral perch before and after remediation of a PCB contaminated lake: Jarnsjon. *Ambio* **27**: 418-424.

GERHARDT, V. AND PUTZGER, J. (1999) Regensburger Leuchtbakterientest- Ein Online Biotestverfahren zur Überwachung von Oberflächengewässern. *Forschungsbericht Umweltbundesamt*.

GRIMME, L. H., ALTENBURGER, R., BACKHAUS, T., BOEDEKER, W., FAUST, M., AND SCHOLZE, M. (1998) Vorhersagbarkeit und Beurteilung der aquatischen Toxizität von Stoffgemischen. **25**: 1-319 *UFZ-Bericht .*

GRIMME,L.H.; ALTENBURGER,R.; BACKHAUS,T.; FAUST,M.; BOEDEKER,W.; SCHOLZE,M. (2000). Kombinationswirkungen von Umweltchemikalien in der Ökotoxikologie. *Umweltwissenschaften und Schadstoff-Forschung* **12** (4): 226- 234.

GUSTAVSON, K.E., SONSTHAGEN, S.A., CRUNKILTON, R.A., AND HARKIN, J.M. (2000). Groundwater toxicity assessment using bioassay, chemical, and toxicity identification evaluation analyses. *Environmental Toxicology* **15**: 421-430.

GUZZELLA L. (1998). Comparison of test procedures for sediment toxicity evaluation with Vibrio fischeri bacteria. *Chemosphere* **37**(14-15): 2895-2909.

HAKSTEGE, A.L. AND VAN GELDERMALSEN, A. (1998). Pilot remediation of sediment from the petroleum harbour in Amsterdam. *Water Science And Technology* **37**: 403-409.

HINWOOD,A.L.; MCCORMICK,M.J. (1987). The effect of ionic solutes on EC50 Values Measured using the Microtox Test. *Toxicity Assessment: An International Quarterly* **2**: 449- 461.

KARUPPIAH, M. AND GUPTA, G.(1996). Impact of point and nonpoint source pollution on pore waters of two Chesapeake Bay tributaries. *Ecotoxicology And Environmental Safety* **35**: 81-85.

KARUPPIAH, M. AND GUPTA, G. (1997). Toxicity of and metals in coal combustion ash leachate. *Journal Of Hazardous Materials* **56**: 53-58.

KISHINO,T.; KOBAYASHI,K. (1995). Relation between toxicity and accumulation of chlorophenols at various pH and their absorption mechanism in fish. *Water Research* **29** (2): 431- 442.

KORNER,S.; DAS,S.K.; VEENSTRA,S.; VERMAAT,J.E. (2001). The effect of pH variation at the

ammonium/ammonia equilibrium in wastewater and its toxicity to Lemna gibba. *Aquatic Botany* **71**(1): 71- 78.

KREBS,F. (1992). Gewässeruntersuchung mit dem durch Alkali- und Erdalkaliionen- Zugabe optimierten DIN Leuchtbakterientest, dargestellt am Beispiel der Saar. *Schriftenreihe des Vereins WaBoLu* 89: 657- 673.

LEBLOND, J.B. AND DUFFY, L.K.(2001). Toxicity assessment of total dissolved solids in effluent of Alaskan mines using 22-h chronic Microtox((R)) and Selenastrum capricornatum assays. *Science Of The Total Environment* **271**: 49-59.

MICHAELIDOU, ST. C., AKKELIDOU, D., AND ZIEGLER, P. (1995). Investigating groundwater pollution from different sources with combined biological and chemical methods. *Science Of The Total Environment* **171**: 51-59.

MIDDAUGH,D.P.; BECKHAM,N.; FOURNIE,J.W.; DEARDORFF,T.L. (1997). Evaluation of bleached kraft mill process water using Microtox(R), Ceriodaphnia dubia, and Menidia beryllina toxicity tests. *Archives Of Environmental Contamination And Toxicology* **32**(4): 367- 375.

MITZ, S. V. AND GIESY, J. P. (1985). Sewage effluent biomonitoring. *Ecotoxicology And Environmental Safety* **10**: 40-52.

PETER,S.; SIERSDORFER,C.; KALTWASSER,H.; GEIGER,M. (1995). Toxicity estimation of treated coke plant wastewater using the luminescent bacteria assay and the algal growth inhibition test. *Environmental Toxicology And Water Quality* **10**: 179- 184.

SAWYER, C.S. AND LIEUALLEN-DULAM, K.K. (1998). Productivity comparison of horizontal and vertical ground water remediation well scenarios. *Ground Water* **36**: 98-103.

SCHMITZ,R.P.H.; EISENTRAGER,A.; DOTT,W. (1998). Miniaturized kinetic growth inhibition assays with Vibrio fischeri and Pseudomonas putida (application, validation and comparison). *Journal Of Microbiological Methods* **31**(3): 159- 166.

SCHMITZ,R.P.H.; KRETKOWSKI,C.; EISENTRAGER,A.; DOTT,W. (1999). Ecotoxicological testing with new kinetic Photorhabdus luminescens growth and luminescence inhibition assays in microtitration scale. *Chemosphere* **38**(1): 67- 78.

WALSH, M.R., WALSH, M.E., AND COLLINS, C.M.(1999). Remediation methods for white phosphorus contamination in a coastal salt marsh. *Environmental Conservation* **26**: 112-124.

WEIß, H., TEUTSCH, G., AND DAUS, B. (1997). Sanierungsforschung in regional kontaminierten Aquiferen (SAFIRA) **27**: 1-205. *UFZ-Bericht*

WEIß, H., TEUTSCH, G., FRITZ, P., DAUS, B., GRATHWOHL, P., TRABITZSCH, R., FEIST, B., RUSKE, R., BOEHME, O. AND SCHIRMER, M.(2001). Sanierungsforschung in regional kontaminierten Aquiferen (SAFIRA) - 1. Information zum Forschungsschwerpunkt am Standort Bitterfeld. *Grundwasser* **6**: 113-122.

WHITEMAN,F.W.; ANKLEY,G.T.; KAHL,M.D.; RAU,D.M.; BALCER,M.D. (1996). Evaluation of interstitial water as a route of exposure for ammonia in sediment tests with benthic macroinvertebrates. *Environmental Toxicology and Chemistry* **15**(5): 794- 801.

Kapitel X

Algal Toxicity of Nitrobenzenes – Combined Effect Analysis as a Pharmacological Probe for Similar Mode of Action

Rolf Altenburger, Heike Schmitt und Gerrit Schüürmann
in Vorbereitung

ALGAL TOXICITY OF NITROBENZENES – COMBINED EFFECT ANALYSIS AS A PHARMACOLOGICAL PROBE FOR SIMILAR MODE OF ACTION

Rolf Altenburger [†], Heike Schmitt [†],Gerrit Schüürmann [†]

[†] Department of Chemical Ecotoxicology

UFZ Centre for Environmental Research Leipzig-Halle

Permoserstraße 15, D-04318 Leipzig

ABSTRACT

An analysis of the toxicity of different nitrobenzenes to the reproduction of the green alga *Scenedesmus vacuolatus* was undertaken. As expected from the literature, a quantitative structure activity relationship correlating log K_{OW} for mononitrobenzenes with their respective EC_{50}-values leads to the assumption that nitrobenzenes may exert narcotic effects as a common type of action, while dinitrobenzenes show a somewhat greater toxicity. Different results are obtained investigating the toxicity of a mixture of 14 nitrobenzenes, which clearly deviates from predicted combined effects as expected for similarly acting compounds on the basis of concentration addition. However, observed effects neither follow those predicted from the concept of independent action. This observation, additional modelling of expectable combination effects for mixed types of action and QSAR indications let us propose that not all of the nitrobenzenes follow the same mode of action. Most interestingly, this result gains combined effect analysis as a pharmacological probe to test for the similarity of mode of action of compound mixtures.

INTRODUCTION

Nitrobenzenes are important environmental chemicals regarding their substantial marketing volumes as industrial chemicals. Their use patterns are diverse ranging from applications as solvents to uses in the synthesis of dyestuffs, urethan polymers, and anilines. Derivative products include various active ingredients of insecticides and herbicides as well as pharmaceuticals.

The aquatic toxicity of nitrobenzenes have been described for unicellular green algae (Deneer et al., 1989, Kramer 1986, Urretarazu Ramos et al. 1999), daphnia (Deneer at al. 1989, Urretarazu Ramos et al. 1999), Tetrahymena (Schultz 1985, Dearden 1995, Schüürmann 1997), photobacteria (Gough 1994), and fish (Deneer 1987, Roberts 1987). Using QSAR techniques for the analysed congeneric compounds lipophilicity has been shown to be a good descriptor of nitrobenzene toxicity in various organisms. For the QSAR of the algal toxicity data hydrogen bonding descriptors in addition to the lipophilicity parameter Ko/w are used. These are interpreted as explainig the polar properties of nitrobenzenes and other compound classes (Deneer et al., 1989, Urretarazu Ramos et al. 1999) while allowing to hold the assumption of narcosis as a common mode of action (Urretarazu Ramos et al. 1999, Vaes et al. 1998). The notion of one comon mode of action of nitrobenzenes in algae has been challenged recently on the basis of QSAR analysis employing E_{LUMO} and E_{SOMO} considerations for the algal toxicities of 19 different nitrobenzenes (Schmitt et al. 2000).

Inherent to a common type of interaction with the biological systems exposed, i.e. polar narcosis for the nitrobenzenes in different aquatic species, is the idea that one compound may act totally or in part as a dilution of the other to eclicit the same effect. Common structural features of chemicals and similar modes of actions are thought to be prerequisites to predict combined effects of chemical mixtures on the basis of the effects of the individual components using the concept of concentration addition (Calamari and Vighi, 1990, Greco and Parsons 1995, Kortenkamp and Altenburger 1998, 1999). In contrast, for mixtures of substances composed of components with different structures and dissimilar modes of action response addition also called independent action is thought to be a valid pharmacological reference concept for toxicity prediction (Pöch 1993). Recent experimental evidence for mixtures of substances with known specific pharmacological mechanisms of action using luminescent bacterial and algal biotests (Altenburger et al. 2000, Backhaus et al. 2000, Faust et al. 2000) strongly support the suitability of the concepts for specifically similarily or dissimilarily acting compounds, respectively.

If the premises of the concepts of concentration addition and response addition are valid even for non-specifically acting compounds at integral levels of toxic effects, it may be possible to use the experimental study of combined effects of mixtures to retrieve information on the type of action of pollutants as proposed by Broderius, Kahl and Hoglund (1995).

The potential of combined effect studies to serve as a pharmacological probe for the similarity of modes of actions of mixture components is the focus of this paper. To this end, we studied the algal toxicity of 19 nitrobenzenes, selected a subgroup of 14 compounds, of which 13 components are described by a simple hydrophobicity-driven QSAR equation while one compound, dinitramine, served as a bait with known dissimilar mode of action and slightly different structure. Combined effect assessment using concentration response relationships for algal reproduction inhibition was then performed by comparing the observed toxicities with

the mixture toxicities calculated on the basis of the single compound toxicities employing the concepts of concentration addition and response addition, respectively.

MATERIALS AND METHODS

Chemicals and reagents
The substances used, sources and purities are specified in table 1.

table 1: Test substances

substance	abbr.	CAS RN	source	charge	purity
Nitrobenzene	NB	98-95-3	Merck	ZA1656639716	99%
2-Chloronitrobenzene	2Cl	88-73-3	Aldrich	CQ03416AQ	>99%
3-Chloronitrobenzene	3Cl	121-73-3	Merck	4316331	>99%
4-Chloronitrobenzene	4Cl	100-00-5	Merck	S00717729	>99%
3-Aminonitrobenzene	3A	99-09-2	Merck	3316702	>99%
4-Aminonitrobenzene	4A	100-01-6	Merck	L344760748	>98%
4-Methylnitrobenzene	4Me	99-99-0	Merck	S01768746	>98%
3,4-Dichloronitrobenzene	3,4Cl	99-54-7	Merck	60108562	>98%
2,3-Diaminonitrobenzene	2,3A	3694-52-8	Fluka	299170690	>97%
5-Chloro-2-aminonitrobenzene	2A5Cl	89-63-4	Merck	K24266586745	>98%
4-Chloro-3-methylnitrobenzene	4Cl3Me	13290-74-9	Fluka	381668/141298	>99%
3-Methyl-4-aminonitrobenzene	4A3Me	99-52-5	Merck	4315751	>98%
Trifluralin	Trif	1582-09-8	Riedel	33340	99%
Dinitramin	Dini	29091-05-2	Riedel	7031851030001	98%
3-Trifluormethyl-4-nitrophenol	TFM	88-30-2	Aldrich	04403K6	99%
2,4,6-Trinitrophenol	2OH1,3,5NB	88-89-1	Merck	K24266586745	>99%
2,4,5-Trichloronitrobenzene*	2,4,5Cl	89-69-0	Riedel	80820	99,5%
1,3-Dinitrobenzene	1,3NB	99-65-0	Merck	K23931047734	>98%
4-Chloro-1,3-dinitrobenzene	4Cl1,3NB	97-00-7	Merck	S03474735	>99%

abbr., abbreviations used in the text; * compound with moistened with approx. 30% water

Test organisms and culture conditions
Liquid cultures of the unicellular green alga *Scenedesmus vacuolatus* Shih. et Krauss, strain 211-15, culture collection Pringsheim (SAG Göttingen, Germany) were grown photoautotrophically at 28 ± 0.5°C in an inorganic, sterilised medium adjusted to pH 6,4 under condition specified earlier (Altenburger et al., 1990). Cells were synchronized by light:dark changes of 14:10 h and a periodic dilution to a standard cell density of 10^6 cells/mL before the onset of the light phase of the growth cycle (t_0). Synchronization was checked by analysis of the cell size distribution at t_0.

Determination of concentration-response relationships
Concentration-response relationships of the test compounds were determined using a 24 h test protocol under synchonized conditions taking the population reproduction (cell number) as effect parameter. The initial cell density was set to 10^5 cells/mL. The culture vessels were composed of tubes with an inner diameter of 1.3 cm and a round bottom. Culture volumes were 8 ml with a headspace of approx. 3 ml. The test medium was the same as for cultivation but enriched with 1.5 mmol/L $NaHCO_2$ providing a final pH of the medium of 7.0.

Illumination was performed by a combination of two types of fluorescent tube lights (L36W/41 Interna, L36W/11 daylight, Osram, Berlin, Germany) with an intensity of 13-18 W/m² (22-33 kLux) providing a photosynthetic active radiation of 350 µE s^{-1} m^{-2}. The aqueous test substances were added to the cultures at t_0. Aliquot samples of the cultures were taken in duplicates at t_0 and at the end of the standard cycle (t_{24}) and the mean cell number was analyzed twice using a CASY II-counter (Schärfe System, Reutlingen, Germany). The inhibition of cell reproduction was calculated by normalizing the data to the results of control cultures. Concentration-response relationships were calculated using either Hill or Weibull analyses, from which the effective concentrations causing a 50% decline in cell reproduction were determined. The concentration-response curves were determined after preliminary range finding testing, striving to provide at least 4 experimental data points between 20 and 80% inhibition. Concentrations of the aqueous stock solutions of the applied chemicals were verified by HPLC-analysis at t_0. A 5 µm reversed phase C8 column (Lichrospher 60 RP select B, Merck, Darmstadt, Germany) or a 5 µm reversed phase C18 column (Lichrospher 100 RP 18-e, Merck, Darmstadt, Germany) were used as stationary HPLC phase, while the mobile phase was for most analyses made up of methanol:water 40:60. All concentration data were corrected for the analytically determined concentration in the test medium at t_0. For the analysis of the parallelity of the concentration-response relationships, all experimental data were linearized using Hill functions and plotted against the logarithm of concentrations.

Mixture toxicity

For the assessment of the mixture toxicity, combination effects observed experimentally in duplicate were compared with the theoretically predicited toxicities, calculated according to the concepts of concentration addition and independent action.

The predictions according to the concept of concentration addition were derived from the calculation of concentrations of single compounds giving a certain effect EC_y. These EC_y values are combined in one multidimensional isobole (equation (Eq. 1)) and this isobole intersected with the mixture ray (equation (Eq. 2)), giving the concentration of the mixture leading to the same effect EC_y (eqation (Eq. 3)).

$$\sum_i \frac{x_i}{EC_{y,i}} = 1 \qquad \textbf{(Eq. 1)}$$

$$a_i = \frac{x_i}{\sum_i x_i} \equiv \frac{x_i}{x_{ka}} \qquad \textbf{(Eq. 2)}$$

$$x_{ka} = \frac{1}{\sum_i \frac{a_i}{EC_{y,i}}} \qquad \textbf{(Eq. 3)}$$

with x_i=concentration of derivative i; $EC_{y,i}$= concentration of the single component i yielding the effect EC_y; x_{ka}=concentration of the mixture as sum of the molar concentrations of the single ingredients; a_i=proportion of component i in x_{ka}.

The predictions according to independent action were derived using the calculated effects of the compounds applied singly at certain concentrations (using their Hill or Weibull functions) and the calculation of their mixture effect according to:

$$y_{ia} = 100 * \left(1 - \prod_{i=1}^{n} \left(1 - \frac{y_i}{100} \right) \right) \quad . \qquad \textbf{(Eq. 4)}$$

with y_{ia}: mixture effect according to independent action; y_i: effect of component i applied singly.

RESULTS

Single compounds toxicities

Exposure of *Scenedesmus vacuolatus* to nitrobenzenes led to a concentration dependent inhibition of cell reproduction (table 2) at concentrations that are well below the water solubility limts of the compounds. The concentration response curves show a clear sigmoid structure demonstrated in figure 1 for the example of 3-methyl-4-chloro-nitrobenzene. The curves were fitted by either Hill functions or Weibull functions, depending on the extent of asymmetry. The EC_{50} values calculated using these concentration response functions cover a range of over 4 log-units (table 2). Concentration values were corrected for analytically determined concentrations in the test medium at the beginning of the experiment (t_0).

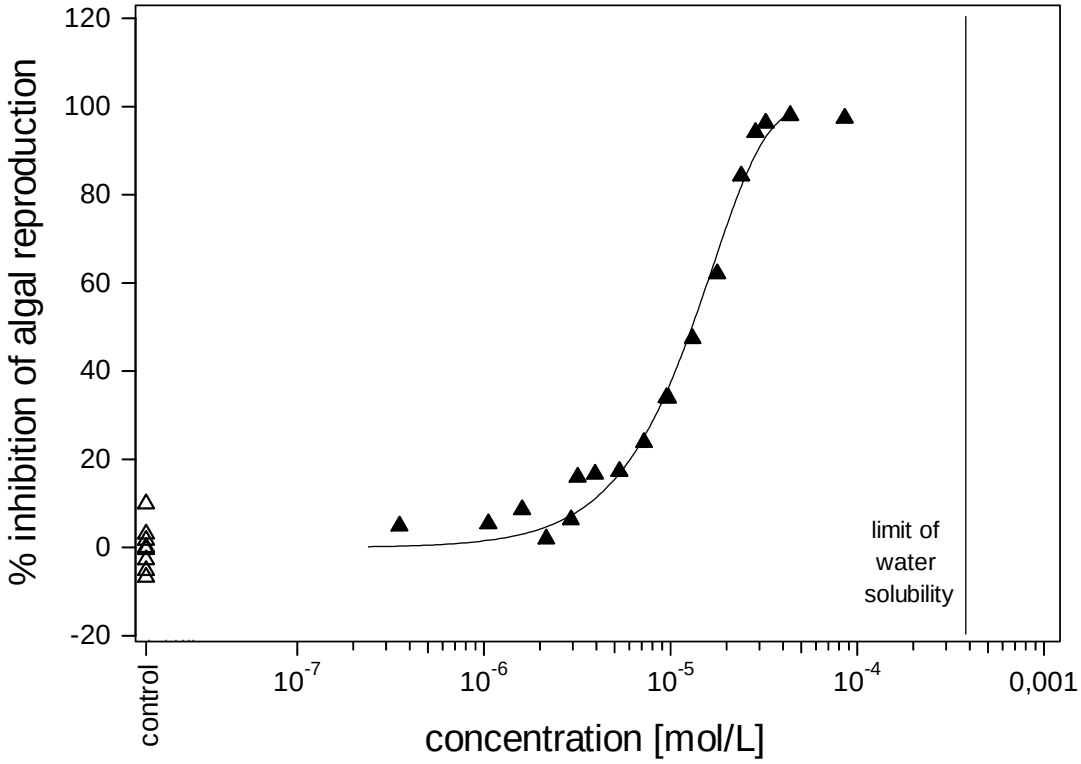


Figure 1: Concentration response data and concentration-response function for 3-methyl-4-chloro-nitrobenzene using a 24 h one generation algal biotest (for details see materials and methods)

table 2: Parameters of the concentration-response curves for nitrobenzenes

substance	log K_{OW} [a]	Hill function [c] x_{50} [mol/l]	p	Weibull function [d] a	b	EC_{50} [mol/l]	EC_{50} [mg/l]
2,4,6-Trinitrophenol	0,89			-14,841	4,757	1,10E-03	252,8
4-Aminonitrobenzene	1,39	3,32E-04	3,04			3,32E-04	45,8
3-Aminonitrobenzene	1,37	2,76E-04	3,22			2,76E-04	38,1
Nitrobenzene	1,85	2,66E-04	4,05			2,66E-04	32,7
2,3-Diaminonitrobenzene	1,27			-6,502	2,574	2,42E-04	37,0
2-Chlornitrobenzene	2,24	1,53E-04	4,72			1,53E-04	24,0
4-Methylnitrobenzene	2,37			-20,552	9,595	1,27E-04	17,4
3-Methyl-4-aminonitrobenzene	1,83	9,04E-05	6,54			9,04E-05	13,7
5-Chloro-2-aminonitrobenzene	2,72			-13,262	7,392	5,55E-05	9,58
4-Chloronitrobenzene	2,39			-4,453	3,046	2,20E-05	3,46
TFM	2,77 [b]			-6,771	5,252	1,66E-05	3,43
3-Methyl-4-chloronitrobenzene	2,90 [b]			-4,171	3,414	1,30E-05	2,23
3-Chlornitrobenzene	2,46			-3,147	3,133	7,72E-06	1,22
4-Chlor-1,3-dinitrobenzene	2,17	2,94E-06	0,89			2,94E-06	0,59
1,3-Dinitrobenzene	1,49	2,49E-06	2,84			2,49E-06	0,42
2,4,5-Trichloronitrobenzene	3,48	1,95E-06	2,79			1,95E-06	0,44
3,4-Dichloronitrobenzene	3,12			-1,242	3,950	1,67E-06	0,32
Trifluralin	5,34	7,28E-08	2,72			7,28E-08	0,024
Dinitramine	4,30	4,59E-08	3,21			4,59E-08	0,015

[a] Hansch, 1995
[b] *CLOGP*, 1999
[c] Hill function: $y = \frac{100}{1 + \left(\frac{x}{x_{50}}\right)^{-p}}$,
[d] Weibull function: $y = 100 * \left(1 - \exp\left(-\exp(a + b\log(x * 10^6))\right)\right)$,
with y: percent inhibition of cell reproduction, and x in mol/l.

In order to assign the nitrobenzenes for which the assumption of a common mode of action could not be rejected, the correlation of toxicity with lipophilicity was investigated. The log Kow values of the compounds (as taken from the literature, table 2) cover a range of 4.5 units which is comparable to the range of the observed toxicities. The correlation and standard deviation of nitrobenzene effects on algae with log Kow were rather poor:

$$- \log EC_{50} = 0,95\ (\pm 0,15) \log K_{OW} + 2,41\ (\pm 0,39) \qquad \text{(Eq. 5)}$$

$$n=19;\ r^2=0,72;\ r=0,85;\ s=0,67;\ F=43$$

On inspection of the residuals, it occurs that three of the four tested dinitrobenzenes may be treated as outliers. The fourth dinitrobenzene, trifluralin, is in good agreement with the QSAR. The neglection of these three substances leads to a QSAR equation of reasonable statistical quality (Schmitt et al. 2000):

$$- \log EC_{50} = 0,96\ (\pm 0,09) \log K_{OW} + 2,15\ (\pm 0,23) \qquad \text{(Eq. 6)}$$

$$n=16;\ r^2= 0,90;\ r=0,95;\ s=0,36;\ F=120$$

Interestingly, trinitrophenol which is nearly totally deprotonated at a pH of 7 as applied in the test, does not deviate from this QSAR although the lipophilic nonionized fraction is much smaller than reflected by the log Kow of the protonated species. The inspection of different effect levels such as EC20 or EC80 does not change the QSAR based on log Kow substantially, apart from the intercept of the equation (data not shown).

A linearization of concentration-response curves according to Hill equations allows for a comparison of the parallelity and steepness of these relationships, as suggested by Chou and Talalay, 1984. Because the focus was set on a comparison of steepness in the area around the EC50 value, only effects between 9% and 91% inhibition were taken into account (-1 – 1 in the linearized Hill plot) (fig 2). Though there is no criteria readily available to judge slopes of different compounds as being non different from parallelism and at the same time allowing for variability, the data analysed here show striking similarity when compared to slope data of known dissimilarly acting compounds (Faust et al, 2000) and even of congeneric and strictly similarly acting compounds (Faust et al. in prep).

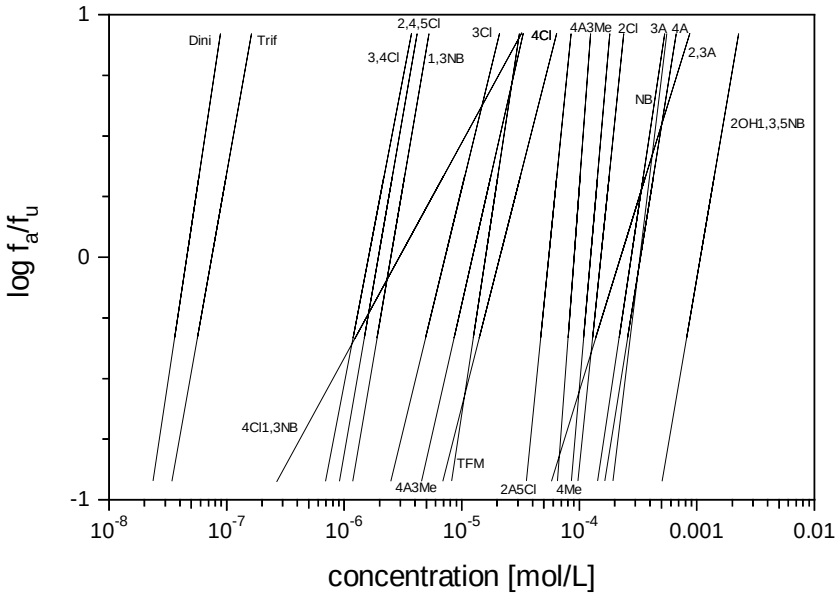


Figure 2: Hill plot to illustrate slopes for biological activities of all 19 nitrobenzenes, Individual substances may be identified by their abbreviation or by using EC50 ordering in table 2.

Mixture toxicity

For the analysis of the combination effect of nitrobenzenes, a mixture of 14 derivatives in proportion of their EC50 values was generated. It comprised of 13 mononitrobenzenes plus the dinitrobenzene dinitramine. The two mononitrobenzenes, namely 2,3-diaminonitrobenzene and 3,4-dichloronitronbenzene were retained due to unsatisfactory variability of responses. Given the reasonable correlation between observed single compounds algal toxicity and lipohilicity (eq. 6) (fig. 3) and their similar curve shape (fig. 2) the selected 13 mononitrobenzenes as mixture components may therefore be expected to show combined effects predictable by concentration adddition. The addition of dinitramine has a clearly distinguishable trait. The compound is a dinitrobenzene derivative with a described specific effect on microtuble assembly from tubulin dimers in plants (Ellis Taylor and Hussey 1994) which is taken to explain its herbicidal properties via mitosis interuption. This mode of action is not even membrane related and might therefore be taken as sufficient evidence to consider it in the context of the mixture as dissimilarly acting.

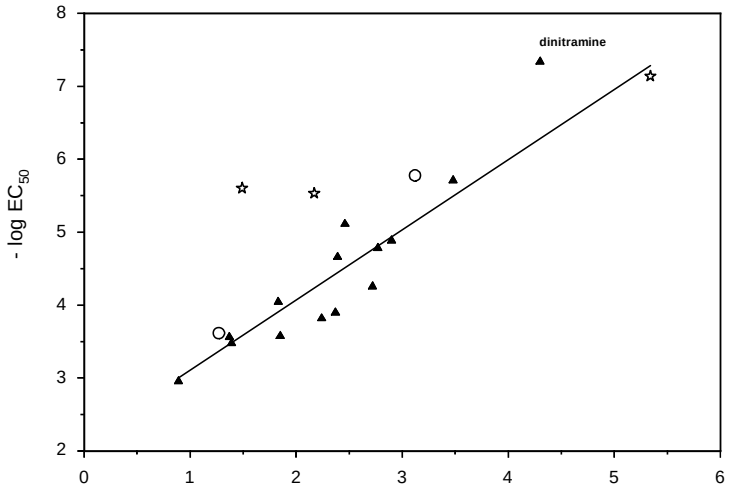


Figure 3: Mixture components and correlation of the algal toxicity of 19 nitrobenzenes with lipophilicity. Solid triangles: mononitrobenzenes selected for the mixture experiment including dinitramine; open symbols: left out compounds, stars: dinitrobenzenes, circles: mononitrobenzenes; line: QSAR according to eq. (6).

The results of the subsequent mixture experiments and predicted combined effects for various assumptions according to the concepts of concentration addition and independent action are displayed in figure 4.

The observed mixture toxicity neither corresponds with the expectations calculated for a concentration additive combination effect nor with an independent effect for all 14 compounds, but lies almost midway between the predictions derived from the two concepts. The calculation of errors of both the experimental and predicted data is not straigthforward, but estimations based on errors determined for repeated single substance testing in the biotest used (Faust et al, 1992) show that the discrimination between both models should be far more than enough to exclude experimental error. The experimental data would only come close to either of the predictions, if 9 of the 14 single toxicities would include a systematic one sided error of 100%.

DISCUSSION

The algal toxicities of 8 of nitrobenzenes reported in this paper are pretty well in accordance with data produced by Kramer et al. 1986 and Deneer et al. 1989 despite the different species and exposure regimes that have been used (*Chlorella vulgaris*, 6 h exposure in Kramer et al.; *Chlorella pyrinoidosa*, 96 h exposure in Deneer et al. 1989).

Similar narcotic mode of action

The QSAR for the algal toxicity of nitrobenzenes given in (6) compares well to the one of Deneer et al. (1989) for mononitrobenzenes (- $log\ EC_{50} = 0{,}90\ log\ K_{OW} + 2{,}03$). Thus on the basis of these findings there is no reason to raise doubts about a common narcotic mode of action of at least mononitrobenzenes in algae.

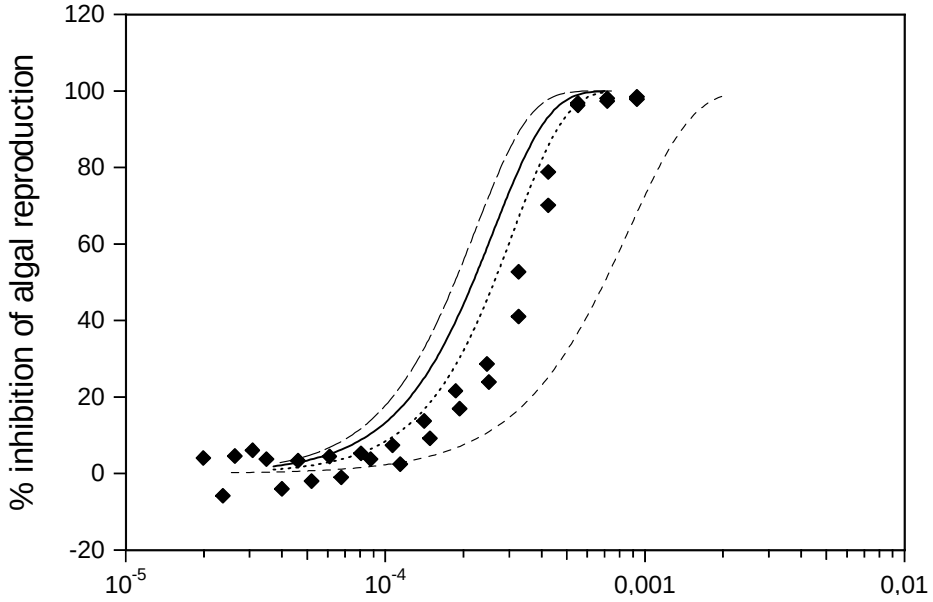


Figure 4: Mixture toxicity of 14 nitrobenzenes compared to the expected combination effects as derived from concentration addition and independent action. diamonds, experimentally observed algal toxicities; long dashed line (_ _) expected concentration additive effects for all 14 compounds; solid line (___) dinitramine independently acting with the 13 other nitrobenzenes; dotted line (.....) dinitramine, TFM and 2,4,5-trichloronitrobenzene indenpendently acting with 11 other nitrobenzenes; short dashed line (- - -) all compounds independently acting.

Parallelity of concentration-response relationships

Parallelism of dose-response curves of components has been interpreted in terms of beeing an indicator of similar biological action and thus a predictor of concentration additive mixture effects in combined effect assessment (Sühnel 1998). In our case, a Hill-linear comparison of the parallelity of the concentration-effect curves of the single compounds shows that the steepnesses of all curves do not deviate substantially except for 1,3-dinitrobenzene and 2,3-diaminonitrobenzene. The former also shows outlying behaviour in the QSAR and both compounds were not included in the mixture experiment. Therefore, again there is no reason to challenge the notion of a similar mode of action of nitrobenzenes in algae. Interestingly, not all of the outliers as identified in the QSAR show a deviating steepness, while a mononitrobenzene, 2,3-diaminonitrobenzene, stands out due to its larger steepness.

Mixture toxicity

Our finding that the algal mixture toxicity of 14 nitrobenzenes is not well predicted by concentration addition is a surprise and stands in contradiction to the interpretation of the QSAR and the single concentration response curves. Könemann (1981) and Hermens and coworkers (1984a,b, 1985) have shown several times for different organisms, that for mixtures of substances with a narcotic type of action concentration addition provides good predictions for expectable mixture toxicities, although no comparison with the predictions for independent action were undertaken. For multiple mixtures of specifically acting substances (pesticides) using algal and bacterial biotests it has been demonstrated that concentration addition allows accurate prediction for mixtures of similarly acting components (Faust et al. in press, Altenburger et al. 2000) while for mixtures composed of strictly dissimilarly acting

chemicals the same holds true for independent action (Faust et al. in press, Backhaus et al. 2000). Therefore, possible reasons for the occurrence of a combined action corresponding to neither of the concepts will be discussed at first.

In the literature, deviations from the concept of concentration addition for substances supposed to be similarly acting are sometimes found, but are not given special attention or classification as synergistic or antagonistic behaviour (Hermens, 1985b, Broderius, Kahl, 1985). If in our case synergistic or antagonistic effects were present, it would not be possible to differentiate between a concentration additive mixture toxicity with antagonistic interference and an independently acting mixture with synergistic interaction. Besides, even for mixtures being in good agreement with one of the concepts, the possibility of synergistic or antagonistic effects based on the contrasting concept cannot be ruled out. On the other hand, there is no obvious pharmcological reason to believe in any type of non-additive interaction for the nitrobenzene mixture components.

More relevant, however, might be to consider the involvement of different modes of action, i.e. some components of the mixture may act similarly and some may not. From the mixtures composition we known that dinitramine has a strikingly different trait being identified as a microtubuli assembly interuptor and thus mitosis inhibitor (Ellis Taylor, Hissey 1994). The calculated expectable combined effects when calculated for the 13 mononitrobenzenes based on concentration addition and for dinitramine in addition on response addition differ from what is expected for all 14 based on concentration addition by about 18% on the concentration scale. This is visible in in terms of a slight shift of the concentration response curve (fig 4), however it does not explain the observed combined effects.

If it is assumed that more nitrobenzenes do deviate from the common narcotic mode of action, the question arises how many and which. Again, several hypotheses seem reasonable. First, several other investigations in nitrobenzenes found differences in the behaviour of derivatives with different substitution patterns. While Kramer & Trümper (1986), Dearden et al. (1995) and Schüürmann Flemmig et al (1997) noted a deviating behaviour of para-substituted derivatives regarding their toxicity to *Chlorella vulgaris* and *Tetrahymena pyriformis*, respectively, Hall Kier (1986) and Bailey and Spanggord (1983) found different effects when comparing ortho- and para- with meta-substituted nitrobenzenes in fathead minnows. From the inspection of residuals of our QSAR, no conclusion can be drawn regarding a systematic deviation of either meta or para derivatives, though. Inclusion of quantum chemical descriptors and in particular comparison of the energy of the singly occupied molecular orbital, E_{SOMO}, of the radical anions as initial metabolites for the nitrobenzene effects under discussion led to the suggestion that some of the compounds may also exert oxidative stress (Schmitt et al. 2000). The potential for redox cycling is tentatively proposed to apply for three of the mixture components, namely TFM, 2,4,5-trichloronitrobenzene and dinitramine. If we now recalculate expectations for combined effect under the assumtion that these compounds act dissimilarly, while the remaining 11 mononitrobenzenes act according to concentration addition we obtain an altered reference curve as depicted in figure 4. In terms of expected concentrations neccessary to evoke the same effect an increase of about 40 % can be calculated and the shift in the predicted concentration response curve clearly goes towards the observed effects. Still there remains an unresolved difference that might indicate that futher dissimilar modes of action of nitrobenzenes might occur.

Mixture toxicity vs. QSAR and parallel concentration effect curves
The QSAR results and the strikingly similar concentration-response curves suggest one common mode of action (narcosis), while the deviation from concentration additive mixture toxicity can be caused by various reasons, not all of them indicating a similar mode of action.

It has to be stressed that the definition of "similar mode of action" may be crucial for the comparison of mixture toxicity experiments with QSAR techniques. Although the terms of mode of action do also vary widely in between the mixture toxicity and the QSAR context, two contrasting approaches may be highlighted. On the one hand, e.g. Pöch (1993) regards a primary, specific, reversible and competitive interaction with one identical molecular receptor as a necessary prerequistite for substances to behave concentration additive, i.e. act similarly. On the other hand, the existence of one common QSAR alone is regarded as sufficient evidence for similar action (Könemann, 1981). It may therefore be possible that the nitrobenzenes act similarly according to a QSAR definition of mode of action, but dissimilarly when looked upon in the mixture toxicity context.

One explanation for the different results regarding the homogeneity of the modes of action as concluded from a QSAR and from mixture toxicity experiments might be derived from the logarithmic transformation of both the concentration and the effect scale for QSAR analyses. It is possible that this reduction of the log-normal concentration scale of the combination toxicity experiments leads to a loss in sensitivity towards systematic deviations. Therefore, a dissimilar behaviour of some components as discussed above might not be visible in a simple one parameter QSAR.

A second explanation regards the variety of processes involved in the toxic effect which cannot be separated in the QSAR approach. If for example a toxifying biotransformation step yields a product which is again quickly biotransformed to a less toxic substance, both processes act in opposite directions regarding the "overall" toxicity as judged by the QSAR. This substance therefore might not be detected as deviating from the QSAR although its mode of action is not similar to that of nitrobenzenes which do not undergo biotransformation.

It remains to be stated that the debate is still open whether results of mixture toxicity experiments may be used for the assignment of substances to modes of action. As detailed knowledge of the molecular mechanisms of toxicities is often lacking, no proof for the possibility of a conclusion from mixture toxicities to modes of action, neither generally nor specifically, has yet been given. Up to now, only the opposite direction of conclusions, i.e. from modes of action to mixture toxicity, seems to be reasonably established.

In summary, an analysis of the toxicity of nitrobenzenes to the green alga *Scenedesmus vacuolatus* was undertaken. As expected from the literature, a QSAR for mononitrobenzenes leads one to assume that nitrobenzenes may exert narcotic effects as a common type of action, while dinitrobenzenes show greater toxicity. Different results are obtained investigating the toxicity of a mixture of nitrobenzenes, which neither follows the predicted effects derived from the concept of concentration addition nor the concept of independent action. This observation might give reason to use combined effect analysis as a mean to pharmacologically probe for the plausibility of a common mode of action of mixture components.

LITERATURE

Altenburger R, Bödeker W, Faust, M Grimme H. 1990. Evaluation of the isobologram method for the assessment of mixtures of chemicals, *Ecotoxicol Environ Safety* 20:98-114

Altenburger R, Backhaus T, Boedeker W, Faust M, Scholze M, Grimme LH. accepted. Predictability of the toxicity of multiple chemical mixtures to Vibrio fischeri: Mixtures composed of similarly acting chemicals. *Environ Toxicol Chem* 19:2341-2347

Backhaus T, Altenburger R, Boedeker W, Faust M, Scholze M, Grimme LH. accepted. Predictability of the toxicity of a multiple mixture of dissimilarily acting chemicals to Vibrio fischeri. *Environ Toxicol Chem* 19:2348-2356.

Bailey H, Spanggord R. 1983. The relationship between the toxicity and structure of nitroaromatic chemicals. *Aquatic Toxicology and Hazard Assessment ASTM STP* 802:98-107

Broderius SJ, Kahl MD. 1985. Acute toxicity of organic chemical mixtures to the fathead minnow. *Aquat Toxicol* 6:307-322

Broderius SJ, Kahl MD, Hoglund MD. 1995. Use of joint toxic response to define the primary mode of toxic actionn for diverse industrial organic chemicals. . *Environ Toxicol Chem* 14:1591-1605

Calamari D, Vighi M. 1992. A proposal to define water quality objectives for aquatic life for mixtures of chemical substances. *Chemosphere* 25:531-542

Chou T-C, Talalay P. 1984. Quantitative analysis of dose-effect relationships: The combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* 22:27-55

CLOGP, version 4.61 1999. Daylight Chemical Information Systems, Irvine, CA.

Cronin M, Dearden J. 1995. Review - QSAR in toxicology. 1. Prediction of aquatic toxicology. *Quantitative Struc-Activit relationship* 14:1-7 XXXXX

Dearden JC, Cronin MTD, Schultz TW, Lin DT. 1995. QSAR study of the toxicity of nitrobenzenes to *Tetrahymena pyriformis*. *QSAR* 14:427-432

Deneer JW, Sinnige TL, Seinen W, Hermens JLM. 1987. Quantitative structure-activity relationships for the toxicity and bioconcentration factor of nitrobenzene derivatives towards the guppy (*Poecilia reticulata*). *Aquatic Toxicology* 10:115-129

Deneer JW, van Leeuwen CJ, Seinen W, Maas-Diepeveen JL, Hermens JLM. 1989. QSAR study of the toxicity of nitrobenzene derivatives towards *Daphnia magna*, *Chlorella pyrenoidosa* and *Photobacterium phosphoreum*, *Aquat Toxicol* 15:83-98

Ellis JR, Taylor R, Hussey PJ. 1994. Molecular modeling indicates that two chemically distinct classes of anti-mitotic herbicide bind to the same receptor site(s). *Plant Physiol* 105:15-18.

Faust M, Altenburger R, Bödeker W, Grimme, LH. 1992. Algentoxizitätstests mit synchronisierten Kulturen. In XXX Autor, *Schriftenreihe WaBoLu 89*, Gustav-Fischer-Verlag, Stuttgart, Germany, pp 311-321

Faust M, Altenburger R, Backhaus T, Boedeker W, Scholze M, Grimme LH. in press. Predicitve Assessment of the aquatic toxicity of multiple chemical mixtures. *J Env Qual*

Greco W, Bravo G, Parsons JC. 1995. The search for synergy: A critical review from a response surface perspective. *Pharmacol Rev* 47:331-385.

Hall LH, Kier LB. 1986. Structure-Activity Relationship studies on the toxicities of benzene derivatives: II. An analysis of benzene substituent effects on toxicity. *Environ Toxicol Chem* 5:333-33

Hermens J, Broekhuyzen E, Canton H, Wegman R. 1985. Quantitative Structure activity relationships and mixture toxicity studies of alcohols and chlorohydrocarbons: Effects on growth of Daphnia Magna. *Aquatic Toxicology* 6:209-217

Hermens J, Canton H, Janssen P, De Jong R. 1984. Quantitative structure-activity relationships and toxicity studies of mixtures of chemicals with anaesthetic potency: acute lethal and sublethal toxicity to Daphnia magna. *Aquat Toxicol* 5:143-154

Hermens J, Canton H, Steyger N, Wegman R. 1984. Joint effects of a mixture of 14 chemicals on mortality and inhibition of reproduction of Daphnia magna. *Aquat Toxicol* 5:315-322

Hermens J, Leeuwangh P, Musch A. 1984. Quantitative structure-activity relationships and mixture toxicity studies of chloro- and alkylanilines at an acute lethal toxicity level to the Guppy (Poecilia reticulata). *Ecotox Environ Safety* 8:388-394

Hermens J, Leeuwangh P, Musch A. 1985b. Joint toxicity of mixtures of groups of organic aquatic pollutants to the Guppy (*Poecilia reticulata*). *Ecotox Environ Safety* 9:321-326

Könemann H. 1981. Fish toxicity tests with mixtures of more than two chemicals: A proposal for a quantitative approach and experimental results. *Toxicology* 19:229-238

Kortenkamp A, Altenburger R. 1998. Synergisms with mixtures of xenoestrogens - a reevaluation using the method of isoboles. *Sci Tot Environ* 221:59-73

Kortenkamp A, Altenburger R. 1999. Approaches to assessing combination effects of oestrogenic environmental pollutants. *Sci Tot Environ* 233:131-140.

Kramer CR, Trümper, L, Berger L. 1986. Quantitative Struktur-Wirkungs-Beziehungen für die Hemmung des autotrophen Wachstums synchroner *Chlorella vulgaris*-Kulturen durch monosubstituierte Nitrobenzene, *Biochem. Physiol. Pflanzen* 181:411-420

Pöch G. 1993. *Combined effects of drugs and toxic agents*. Springer Verlag, Wien, Austria

Roberts DW. 1987. An analysis of published data on fish toxicity of nitrobenzene and aniline derivatives. In Kaiser KLE, ed, *QSAR in Environmental Toxicology - II*, D Reidel, Dordrecht, Netherlands, pp 295-308

Schmitt H, Altenburger R, Jastorff B, Schüürmann G. 2000. Quantitative structure-activity analysis of the algae toxicity of nitroaromatic compounds. *Chem Res Toxicol* 13:441-450.

Schüürmann G, Flemmig B, Dearden JC, Cronin MT, Schultz TW. 1997. CoMFA study of acute toxicity of nitrobenzenes to *Tetrahymena pyriformis*. In Chen F, Schüürmann G, eds, *QSAR in environmental sciences VII*, SETAC Press, Pensacola, FL, USA, pp 315-328

Schultz T W, Moulton B A. 1985. Structure-activity relationships for nitrogen-containing aromatic molecules. *Environ Toxicol Chem* 4:353-359.

Sühnel J. 1998. Parallel dose-response curves in combination experiments. *Bull Mathematical Biology* 60:197-213

Vaes W H J, Urrestarazu Ramos E, Verhaar H J M, Hermens J L M. 1998. Acute toxicity of nonpolar versus polar narcosis: Is there a difference? *Environ Toxicol Chem* 17:1380-1384.

Urrestarazu Ramos E, Vaes W H J, Mayer P, Hermens J L M. 1999. Algal inhibition of *Chlorella pyrenoidosa* by polar narcotic pollutants: toxic cell concentrations and QSAR modeling. *Aquat Toxicol* 46:1-10